DISC1 & GSK3β modulate PDE4 activity: Functional integration of psychiatric associated signalling pathways

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PhD

The University of Edinburgh

2010

Declaration

I declare that, except for where noted, all work contained in this thesis was performed and composed by myself. Where others have contributed to elements of the work, this is stated clearly in the text. No element of this work has been submitted for any other degree of professional qualification.

Becky C. Carlyle

Acknowledgements

Well, it has been a tough almost 4 years, but without the guidance of my supervisors David Porteous & Kirsty Millar I would never have made it. They have been a constant source of both practical and emotional help, and for this I am extremely grateful. I would also like to thank the Edinburgh University College of Medicine & Veterinary Medicine for providing my PhD Scholarship.

It has been wonderful to be a part of the DISC1 clan for the last three years, a group of interesting, supportive and highly knowledgeable people. Sheila Christie, Shaun Mackie, Christoph Grünewald, Nick Bradshaw, Fumiaki Ogawa and Elise Malavasi have all been incredibly patient and provided plenty of laughs along the way. I feel very lucky to have been a part of this team, and I hope to keep in touch with everyone there when I leave. Outside of the DISC1 group there are too many people to mention who have all been keen to help me out at some point: Laura, Abby, Rosemary, Susan, Heather, Helen, Barbie, Pippa, Lowri, Justyna…I'm sure I must have missed more. The MMC is a fantastic place to work, and I will miss everyone I have had the pleasure to work with there.

Outside the MMC, big thanks go to Paul Perry and Bertrand Vernay at the MRC Human Genetics Unit for their guidance with confocal imaging, to Ted Hupp for the use of his EnVision Plate Reader, and to Tony Harmar for early discussions which began my trip down the winding road of pharmacology. I would also like to acknowledge Professor Miles Houslay and Professor Tetsu Akiyama for their kinds gifts of the PDE4B, PDE4D and α-DISC1 antibodies.

The MMC has a fantastic bunch of PhD students, and they have helped ease me through the bad times and enjoy the good times. Sarah, I hope we have the chance to gain another serious hangover in Newington, but this time can we do it without the Radiation Course at the beginning? Ellen, you have been an absolute rock, and your wise (and

somewhat sardonic!) words have kept me going at the darkest times. Gareth, Rosie (and Emma!), your generosity in my time of need will always be remembered, and I hope that PhD karma does the same for you in a couple of years time. Manu, your homemade pizza really wasn't that bad!

And so, to the behind the scenes stars. It all started when this chap from the Physics Department appeared in the booth behind Sheila, who teased me for weeks as I couldn't stop smiling whenever I spoke to him. Two and a bit years down the line, Rob has provided love, support, patience, Oxford commas, and in between getting our PhDs done, we've had a huge amount of fun. I eagerly await our move to the USA, and look forward to the next chapter of our story. Thanks Mister.

To the two Helens and Toast, and all my friends dispersed around the globe, thank you. But most of all, I would like to thank my Mum, Dad, Sister and Pete, for taking it in their stride when I left Clinical Medicine, for always being on the end of the phone with comforting words, for their unerring support and patience, and their excitement at this tiny field of molecular medicine I have devoted the last few years to. I love you all to bits, and this thesis is dedicated to you.

Abstract

Following the discovery of the DISC1 gene in 2000, subsequent research has led to DISC1 becoming one of the most promising candidate genes for psychiatric disorders. Acting as a scaffold protein, DISC1 has a large number of interacting proteins and is involved in a series of intracellular signalling pathways. Amongst these binding proteins are two enzymes, PDE4 and GSK3β, that were originally implicated in psychiatric disease by virtue of their inhibition by psychoactive drugs. PDE4 enzymes are inhibited by rolipram, which possesses anti-depressant and anti-psychotic activity, while GSK3β is one of the major targets of lithium, a potent mood stabiliser. Both these enzymes are intricately involved in the PI3K/AKT, cAMP, and MAPK signalling pathways, all of which have a number of downstream outcomes with potential relevance to psychiatric disorders. The Millar and Porteous laboratory had established that DISC1 modulates PDE4 activity, but this predated awareness of GSK3β as another DISC1 interactor whose binding site overlapped with that of PDE4. Since cAMP is a key regulator of signalling pathways in the brain, I hypothesised that not only DISC1, but also GSK3β may be involved in the regulation of PDE4 activity to control local cAMP levels and gradients.

To investigate this hypothesis, I characterised SHSY5Y cells as a model for measuring PDE4 activity, and performed a series of genetic and pharmacological manipulations on this system. Inhibition of GSK3β resulted in a decrease of basal PDE4 activity that was amplified by DISC1 overexpression. Wild type cells that were treated with forskolin exhibited a significant increase in PDE4 activity, which was suppressed by GSK3β inhibition and both overexpression and knockdown of DISC1. Further experiments confirmed that none of these changes were a result of differences in PDE4 mRNA or protein expression. Thus I have provided evidence that suggests tonic activation of PDE4 by GSK3β and evidence for modulation of PDE4 activity by DISC1. I provide evidence for the localisation of PDE4B & PDE4D with key psychiatric associated

receptors in structures resembling developing dendritic spines; furthermore, agonism of NMDA receptors results in a significant increase in PDE4 activity in primary neurons.

These results are a simple demonstration of an emerging principle in psychiatric research: that none of the signalling pathways implicated in psychiatric disease are acting in isolation. There are likely to be multiple points of integration between these pathways, with the demonstrated DISC1-GSK3β-PDE4 interaction forming one of these points. My results add an important new element to the understanding of how the DISC1 complex may regulate intracellular signalling in response to extracellular cues.

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List of abbreviations used in this thesis

Genes and proteins

AKAP	A-Kinase Anchoring Protein
AMPA	α-amino-3-hydroxyl-5-methyl-4isoxazole propionate
APE	AKT Phosphorylation Enhancer
ATF	Activating Transcription Factor
B2M	Beta-2 microglobulin
Bcl-2	B-cell lymphoma-2
BDNF	Brain Derived Neurotrophic Factor
cAMP	Cyclic Adenosine Monophosphate
CBP	CREB Binding Protein
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CREB	cAMP Response Element Binding
DAT	Dopamine Transporter
DISC1	Disrupted in Schizophrenia-1
DOPA	3,4-dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
DRD	Dopamine Receptor
EIF3	Eukaryotic Initiation Factor
ERK	Extracellular Related kinase
FEZ1	Fasciculation of Elongation Factor Zeta 1
GABA	Gamma Aminobutyric Acid
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GBP	GSK3β binding protein
Grb2	Growth Factor Receptor-Bound Protein 2
GRIN2B	Glutamate Receptor Subunit Epsilon-2
GSK3β	Glycogen Synathase Kinase 3 Beta
HCN	Hyperpolarisation Activated Cyclic Nucleotide Gated channel

HSP70	Heast Shock Protein 70
HVA	Homovanillic Acid
IP3	Inositol trisphosphate
JNK	C-Jun N-terminal kinase
KIF5A	Kinesin Heavy Chain Isoform 5A
LIS1	Lissencephaly 1
MAP2	Microtubule Associated Protein 2
MBP	Myelin Basic Protein
mTOR	Mammalian Target of Rapamycin
NDE1	Nuclear Distribution Factor Element 1
NDEL1	Nuclear Distribution Factor Element Like 1
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartic Acid
NRG1	Neuregulin 1
NT-3	Neurotrophin-3
PDE4	Phosphodiesterase-4
PSD95	Post Synaptic Density 95
PIP3	Phosphatidylinositol (3,4,5)-Trisphosphate
PKA	Protein Kinase A
PPIA	Peptidylprolyl Isomerase A
PTEN	Phosphatase and Tensin Homolog
RGS4	Regulator of G protein signalling
RPLPO	Large Ribosomal Protein
SPTAN1	Spectrin Alpha Chain 1
TSNAX	Translin-Associated Factor X
5’AMP	5’ Adenosine Monophosphate

Units

^{o}C	Degrees Centigrade
Da	Dalton
g	Gram
l	Litre
m	Metre
M	Molar
rpm	Revolutions per minute
V	Volt

Amino Acids

A	Alanine
C	Cysteine (also written as Cys)
F	Phenylalanine (also written as Phe)
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
P	Proline
Q	Glutamine
R	Arginine
S	Serine (also written as Ser)
T	Tyrosine (also written as Tyr)
W	Tryptophan
p	Indicates phosphorylation of the residue/protein

Nucleotides

A	Adenine
C	Cytosine
G	Guanine
T	Thymidine

Other abbreviations

2 xYT	Yeast Extract Tryptone Medium
AMV	Avian Myeloblastosis Virus
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
BRET	Bioluminescence Resonance Energy Transfer
BSA	Bovine Serum Albumin
CA1	Cornu Ammonis
CAR	Conditioned Avoidance Response
CATIE	Clinical Antipsychotic Trials of Intervention Effectiveness
cDNA	Complementary DNA
CMV	Cytomegalovirus
CNG	Cyclic Nucleotide Gated
CNV	Copy Number Variation
CO_2	Carbon Dioxide
CRUK	Cancer Research United Kingdom
C-terminus	Carboxy Terminus
CT	Cycle Threshold
CTP	Cytidine Triphosphate
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4’6-diamidino-2-phenylindole
dH_2O	Deionised water

d.i.v.	Days *in Vitro*
DLPFC	Dorso-lateral Pre Frontal Cortex
DMEM	Dulbecco Modified Eagl's minimum essential Medium
DMSO	Dimethly Sulfoxide
DNA	deoxyribonucleic acid
DSM-IV	Diagnostic and Statistical manual of Mental Disorder (Fourth Edition)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ENU	N-nitroso-N-ethylurea
EPS	Extra Pyramidal Symptoms
E18	Embryonic day 18
Es	Extremely Short Isoform (of DISC1)
Esv	Extremely Short Variant Isoform (of DISC1)
FACS	Fluorescence Activated Cell Sorting
FL	Full length
FRET	Fluorescence Resonance Energy Transfer
GTP	Guanosine Triphosphate
GPCR	G Protein Coupled Receptor
GWAS	Genome Wide Association Study
GST	Glutathione S-transferases
HARBS	High Affinity Rolipram Binding Site
HBSS	Hank's Buffered Salt Solution
HEK293	Human Embryonic Kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCl	Hydrochloric Acid
H89	N-[2-(p-Bromocinnamylamino)ethyl]-5-5soquinolinesulfonamide.2HCl
IBMX	Isobutylmethylxanthine
ICC	Immunocytochemistry
IC50	Half Maximal Inhibitory Concentration
IP	Immunoprecipitation

Ki	Dissociation constant for inhibitor binding
L	Long isoform (of DISC1)
LARBS	Low Affinity Rolipram Binding Site
Lv	Long Variant isoform (of DISC1)
LB	Lysogeny Broth
LEF/TCF	Lymphoid Enhancer Factor/T-cell Factor
LiCl	Lithium Chloride
LOD	Logarithm of Odds
LSM510	Laser Scanning Microscope 510
LTD	Long Term Depression
LTP	Long Term Potentiation
MAPK	Mitogen Activated Protein Kinase
mEPSC	Miniature Excitatory Post Synaptic Current
Mg^{2+}	Magnesium ion
mRNA	Messenger RNA
MRI	Magnetic Resonance Imaging
NaCl	Sodium Chloride
NaFl	Sodium Fluoride
NaOH	Sodium Hydroxide
NLS	Muclear localisation signal
N-terminus	Amino Terminus
PBS	Phosphate Buffered Saline
PCP	Phencyclidine
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PFC	Pre Frontal Cortex
PI3K	Phosphoinositide 3-kinase
PPI	Prepulse Inhibition
P1	Post natal day 1
RACE	Rapid Amplification of cDNA Ends

RNA	Ribonucleic Acid
RT-PCR	Reverse-transcriptase PCR
S	Short isoform (of DISC1)
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium Dodecyl Sulphate
SNP	Single Nucleotide Polymorphism
TBE	Tris Borate EDTA
TDZD-8	Thiadazolidinone
Tris	Tris(hydroxymethyl)aminomethane
TTP	Thymidine triphosphate
UCR	Upstream Conserved Region
WCST	Wisconsin Card Sort Test

1 Introduction

1.1 Introduction to Schizophrenia

Schizophrenia is a severe chronic psychiatric disorder with a lifetime risk of 1% (for a comprehensive review see Owen et al., 2005), carrying a total cost to English society estimated at £6.7 billion per year. £2 billion of this figure accounts for direct treatment and care by the NHS (Mangalore R, 2007). The incidence of the disease is increased in minority migrant populations and urban areas (McGrath et al., 2004). Prevalence of the disease is believed to be greater in males than females throughout most of adulthood, with rates equalising by the end of the disease risk period, though this may be due to diagnostic bias (Aleman et al., 2003). The disease is characterised by two forms of symptoms, which can be loosely classified as "positive," including hallucinations, delusions and formal thought disorder, and "negative" symptoms of withdrawal, loss of volition, affective flattening and poverty of speech. Underlying these deficits and predating their onset is a persistent "core" cognitive disorder (Rund, 1998) which correlates with daily living deficits in 50% of Scottish cases studied (Jablensky, 2006). These cognitive deficits affect working memory, short term and episodic memory, attention, and executive function, and do not alter in magnitude during psychotic episodes or periods of remission. A subgroup of first degree relatives demonstrate a similar pattern of cognitive deficits to their affected relatives, marking these measurable traits as possible intermediate traits or "endophenotypes" (Asarnow et al., 2002) to aid genetic analysis, the rationale being that these "simpler" quantitative phenotypes may prove easier to link to genetic variation then the heterogenic entity of schizophrenia (Gottesman and Gould, 2003).

The aetiology of schizophrenia is still largely unknown, since it is a complex disorder with multiple potential susceptibility genes and environmental risk factors. Although schizophrenia is highly heritable, the genetic architecture is complex, with no simple pattern of inheritance and no common genes of major effect (Sullivan et al., 2003). Despite a clear genetic influence, concordance in monozygotic twins is still only 50%, suggesting an important interaction of underlying genetic susceptibility with environmental factors (Sullivan et al., 2003).Where the balance lies between rare mutations of large effect and common variants of small effect is the subject of active debate (McClellan et al., 2007, Craddock et al., 2007), which will only be resolved by experimental evidence from candidate gene studies, high resolution linkage studies in high risk families, genome wide studies of single nucleotide polymorphisms (Consortium, 2009) and copy number variation (Sklar and Consortium, 2008, St Clair, 2008) with follow-through gene resequencing and functional studies. Indeed, several studies have now shown that affected individuals are more likely to harbour rare structural DNA variants than healthy controls (Xu et al., 2008, Walsh et al., 2008). The process by which an association with a disease can be confidently proven was originally defined by A.B. Hill, with reference to epidemiological studies of environment and disease (Hill, 1965), but can be applied to modern genetics studies. An undoubtable association must be strong and consistent across studies, be specific to the disease, expressed at the appropriate time, possess a biological gradient of effect, have biological plausibility that does not contradict established knowledge, and finally, show effects consistent with association in experimental situations (Hill, 1965). Despite any single causative factor in psychiatric genetics that can fulfil all of these traditional criteria, the appearance of multiple candidate genes over the last decade has led to biological pathways of interest for the disease, which will be crucial to advance our full understanding of this highly complex disorder (Porteous, 2008).

With significant advances in ascertaining genes of interest in relation to schizophrenia in the last 20 years, the old question of the Kraepelinian divide has been re-addressed. In the early 20th century, Emil Kraepelin posited that dementia praecox, (which evolved

into the entities we know today as psychosis and schizophrenia), and manic depression (bipolar disorder), were two distinct diseases (Craddock and Owen, 2005). Bipolar disorder is a disease characterised by the experience of one or more manic episodes, during which patients can experience an excess of energy, a lack of need for sleep, inflated self esteem, and an increased participation in potentially risky behaviour. These manic episodes are interspersed with periods of depression (hypomania), where a patient can experience a range of symptoms including anhedonia, fatigue, insomnia, weight loss and a diminished ability to concentrate (DSM IV diagnostic criteria). Despite the fact that the symptoms of these two conditions often overlap, and can be different to confidently diagnose, the notion of this diagnostic divide has continued. It is only in the last 10 years, with the availability of information about the genetics underlying the susceptibility to these two conditions, that the aetiology increasingly appears to share common features. In a large Scottish family, covered in greater detail below, where disruption of the gene DISC1 is believed to be a causal event, there are cases of not only bipolar disorder and schizophrenia, but also major depression (Blackwood et al., 2004). Association studies on this same gene have shown multiple associations with bipolar disorder and schizophrenia in different populations (see Chubb et al., 2008 for a detailed review). Whole genome scans of both disorders highlight some common chromosomal regions of susceptibility (Ferreira et al., 2008, Green et al., 2009), while standard association studies reveal that the D-amino oxidase activator gene and NRG1 are associated with both disorders (reviewed Craddock et al., 2005). A spectacularly large Swedish family study using registries encompassing the entire Swedish population finds that first degree relatives of probands with schizophrenia have an increased risk of developing bipolar disorder, and vice versa (Lichtenstein et al., 2009). This landmark study not only suggests some shared genetic aetiology for the diseases, but also suggests a small impact of shared environmental features. With this rapidly progressing evidence, there is a large movement to start viewing psychiatric disorders as a spectrum, perhaps with multiple dimensions. Where a patient is placed on this spectrum may be dictated by the particular combination of risk alleles they possess, and their exposure to environmental sources of risk. This concept will be revisited throughout this thesis, as

evidence from studies of bipolar disorder treatment, schizophrenia genetics, and basic molecular biology are combined to try and further dissect signalling pathways that may go awry in these conditions.

1.2 Introduction to DISC1

1.2.1 DISC1 – The Initial Discovery

The story of DISC1, one of the strongest candidate susceptibility gene for schizophrenia began 20 years ago, with the report of a large Scottish family (Stclair et al., 1990) carrying a balanced translocation at (1;11)(q42;q14.3) which associates with various major psychiatric disorders, including schizophrenia (Blackwood et al., 2001). Of the 87 karyotyped family members, 37 carry the translocation, and 29 of these carriers has a psychiatric diagnosis. In the most recent study of this family, 7 of these carriers were diagnosed with schizophrenia, 10 with recurrent major depression, and one with bipolar disorder. There was also one case each of anxiety, alcoholism and minor depression, and two cases of adolescent conduct disorder. Linkage studies gave a maximum LOD score of 7.1 for segregration of the translocation with a broad clinical phenotype encompassing schizophrenia, recurrent major depression and bipolar disorder. All tested carriers of the translocation exhibit a reduced P300 event related potential, a measurement of attention dependant information processing impaired in non translocation carrying schizophrenics (Blackwood et al., 2001). Thus, inheritance of the translocation results in functional brain deficits in all carriers. The translocation breakpoints were cloned and the gene named Disrupted in Schizophrenia-1 identified as directly disrupted by the breakpoint on chromosome 1 (Millar et al., 2000). Also interrupted by the translocation is a non coding RNA gene located antisense to DISC1, named Disrupted in Schizophrenia 2 (Millar et al., 2000).

1.2.2 An overview of DISC1 genetic studies

Studies in the wider population have so far failed to reach a consensus as to a single causative SNP or haplotype in the DISC1 gene, but an ever increasing number of

positive findings in genetic linkage and association studies suggest a role for DISC1 allelic heterogeneity in not only schizophrenia, but also in a wider spectrum of psychiatric diagnoses including bipolar disorder and major depressive disorder (Reviewed in Chubb et al., 2008). It is important to state there have also been negative association findings (Devon et al., 2001, Kockelkorn et al., 2004, Zhang et al., 2005). Since this review, the first round of the CATIE (Clinical Antipsychotic Trials of Intervention Effectiveness, a large scale and multi-faceted schizophrenia study funded by the National Institute of Mental Health and co-ordinated by the University of North Carolina) schizophrenia Genome Wide Association Study (GWAS) shows a trend towards positive results for DISC1, but this result does not reach genome wide significance (Sullivan et al., 2008). There are also positive findings in a GWAS for late onset Alzheimer disease (Beecham et al., 2009). Other GWAS results have proved negative with respect to DISC1 (O'Donovan et al., 2009). Despite this, the weight of positive evidence in favour of DISC1 variants conferring susceptibility to psychiatric disorders in the general population is increasingly convincing.

Association studies concerning neurophenotypes outwith psychiatric disorders are also interesting, suggesting a more generalised role for DISC1 in disease with cognitive deficits or a potential neurodevelopmental component. A Finnish group report evidence that associates a DISC1 haplotype and microsatellite within intron 9 previously associated with schizophrenia, with males with autistic spectrum disorders and Asperger syndrome (Kilpinen et al., 2007). Given the core cognitive disorder present in schizophrenic cases, other groups are working to find potential effects of DISC1 on subclinical measures of cognitive ability. Again this is well reviewed by Chubb *et al*, 2008, but as a short summary, various studies report linkage or association between DISC1 variants and impaired cognitive function in both schizophrenic and normal individuals. Most notably, the HEP haplotypes originally introduced by Hennah (Hennah et al., 2003) are variably associated with reduced pre frontal cortex grey matter, impaired long term memory and impaired working spatial memory (Cannon et al., 2005).

A lot of focus thus far has been on the DISC1 Ser704Cys polymorphism, which associates with schizophrenia, schizoaffective disorder and major depression in various populations (Callicott et al., 2005, Hashimoto et al., 2006, Qu M and Tang F, 2007). Healthy subjects carrying the Cys allele show decreased hippocampal grey matter volume in a North American population (Callicott et al., 2005), and an Italian population (Di Giorgio, 2008)an increased volume of medial superior frontal gyrus and short insular cortex in one Japanese population (Takahashi et al., 2009). A further Japanese population shows decreased grey matter volume in the cingulate cortex, cingulate and posterior gyrus, with reduced white matter integrity in PFC (Hashimoto et al., 2006). Interestingly, this study also reports lower extracellular signal relate kinase (ERK) activity associated with the Cys allele (Hashimoto et al., 2006). In the former Japanese study, schizophrenic Cys carriers have a smaller supramarginal gyrus, and the volume of the right medial superior frontal gyrus correlates with neuroleptic dosage in Ser homozygotes (Takahashi et al., 2009). Finally, the Italian study, in attempting to connect structural differences to function, found that Ser homozygotes show increased hippocampal activity, with enhanced connectivity between the formation and the right middle frontal gyrus during a memory encoding task, despite there being no genotype associated difference in task performance (Di Giorgio, 2008). There are relatively weak indications of a detrimental effect of this allele on normal cognitive ageing in females in a Scottish cohort of subjects (Thomson et al., 2005). It is not yet clear whether this variant is significant in itself, or whether it acts to tag a haplotype responsible for the phenotypes observed.

Recently, the theory that variation in multiple schizophrenia susceptibility genes may interact to heighten the risk of schizophrenia in an individual has become popular. To this end, combinatorial studies involving DISC1 have begun, focusing originally on NDE1 and NDEL1, two well established protein binding partners. The first of these studies shows genome wide linkage to schizophrenia with a marker close to NDE1, when data is conditioned on a background of the previously identified HEP3 DISC1 risk

haplotype. Further study of 4 tagging SNPs within NDE1 produces 2 SNPs with a putative association, but these fail to survive Bonferroni correction (Hennah et al., 2007). In a different approach, a hypothesis driven study finds two major NDEL1 haplotypes, one of which is more commonly transmitted in healthy controls. On conditioning on a background of DISC1 risk (hetero or homozygosity for the Cys allele), there is a significant effect of NDEL1 haplotype (via a tagging SNP, rs1391768) on schizophrenia risk in Ser homozygotes. Conversely, the genotype at rs3784859 in NDE1 shows a putative effect on schizophrenia risk only in combination with a Cys carrying background. This leads to the hypothesis that NDE1/NDEL1 genotype may effect the efficacy of protein binding to DISC1 (Burdick et al., 2008). Finally, an interaction between NRG1 and DISC1 SNPs has been observed: the T/T genotype of rs2793092 in DISC1 only shows an association with increased lateral ventricle volume in schizophrenic patients on a background of their also carrying the T allele of rs6994992 in NRG1 (Mata et al., 2009).

Functional studies of the Ser704Cys SNP suggest that the Cys variant increases the propensity of DISC1 to form oligomers (Leliveld et al., 2009). The variant lies within the oligomerisation region of residues 668-747 as defined by Leliveld *et al*, 2009. Overexpression of the Cys variant results in a subtle decrease of NDEL1 binding in human neuroblastoma cultures in comparison to Ser variant overexpression (Leliveld et al., 2009). This result is in agreement with earlier work which suggested that the Cys variant of DISC1 interacts more strongly with NDEL1 in yeast-2-hybrid screening, and shows a mild trend towards a decreased DISC1-NDEL interaction as measured by co-immunoprecipitation (Kamiya et al., 2006). Thus, a DISC1 variant that is related to psychiatric disease by genetic association may affect both DISC1 function, and the function of a different, interacting protein. This finding may begin to explain the interplay between SNPs in these proteins.

There are three more disparate pieces of evidence to add to the genetic association of DISC1 with psychiatric disease. The first of these derives from a relatively new field of

genetic study: that of copy number variation (CNV). These variations vary in size, and encompass deletions, insertions and duplications of sections of DNA. The first major study published investigating this phenomenon reveals a gain of sequence in DISC1 in a mother and her child, but no psychiatric diagnosis is reported for these subjects (Redon et al., 2006). Further evidence of the presence of CNVs is required to judge the significance of this kind of variation in DISC1. The next piece of evidence is the presence of ultra rare structural variants in DISC1 that may lead to disease. Deep sequencing was performed on likely regions of functional significance in DISC1 in 288 schizophrenia patients and controls. As well as detecting the common variants used in association studies, Q246R, S704C and L607F (the former two of which were weakly associated with schizophrenia in this cohort), six patients are found to carry ultra rare schizophrenia specific mutations (R37W, S90L, T603I, G14A and R418H) not present in a scan of 10,576 control alleles. The authors attribute 2% of the risk for schizophrenia to these ultra rare variants (Song et al., 2008).

The final piece of genetic evidence comes from a large body of work characterising the various isoforms of DISC1 (Nakata et al., 2009). Over 50 full length DISC1 transcripts are identified from post mortem human brain using a combination of PCR, long range PCR and sequencing and 3 and 5' RACE. Expression of transcripts with deletions of exon 7 and 8, and a short variant transcript (Esv) is significantly increased in schizophrenic hippocampi. Furthermore, the expression of the transcript lacking exon 3 is significantly associated with the homozygous A allele at intronic SNP rs821597, and possession of the phenylalanine variant of L607F or homozygosity for the Cys variant of S704C leads to increased expression of the transcript lacking exons 7 and 8 (Nakata et al., 2009). This experiment is exciting, as it links two previously unassociated DISC1 risk SNPs to a potential mechanism, suggesting how structural changes may lead to differential DISC1 expression, and thus pathological physiology. In their earlier study, possession of DISC1 risk variants (C at hCV219779, G at rs821597 and A at rs821616) led to significantly decreased mRNA expression of the DISC1 interacting proteins FEZ1, LIS1 and NUDEL in post mortem hippocampus (Lipska, 2006). Independent of

genotype, mRNA was decreased for each of these three proteins in the hippocampus of schizophrenic patients, and showed a trend towards a mild decrease in the dorso-lateral prefrontal cortex (DLPFC) (Lipska, 2006).

To summarise, the genetic evidence for the potential role of DISC1 in predisposition not only to schizophrenia, but to other major psychiatric and cognitive disorders is increasingly convincing. Up to January 2009, 24 association studies of DISC1 and schizophrenia had been performed – 15 of these reported positive association (Lakhan and Kramer, 2009). Genetic and phenotypic evidence (both from MRI and performance in behavioural tests) suggests a distinct role for DISC1 in normal hippocampal function. The fact that there has been no single causative and biologically explainable finding to account for the role of DISC1 in all patients suggests one of two prospects – either that there is allelic heterogeneity, with multiple variations within the gene resulting in susceptibility in different cases, or that the markers studied thus far are in high linkage disequilibrium with a true causative variant. Evidence thus far, most strikingly the failure to narrow down the area of interest within the DISC1 gene to a specific region, and the new evidence for ultra rare causative variants, suggests that allelic heterogeneity is the more likely explanation. This suggestion has yet to reach full acceptance in the psychiatric genetic community, but is seen in other conditions such as Cystic Fibrosis, where almost 1300 disease causing mutations have been reported in the CFTR gene (des Georges et al., 2004). It is hoped that deep sequencing of DISC1 will add significantly to knowledge in this area.

1.2.3 DISC1 splice variants

The human DISC1 gene contains 13 exons which produce a full length transcript of 7.5kb (Millar et al., 2001). The number of DISC1 isoforms reportedly produced by this transcriptionally complex locus is still under discussion. There are 4 robustly confirmed human isoforms: “Long” (L), encoded by all 13 exons, “Long variant” (Lv) arising from an alternative splice site in exon 11, “Short” (S) arising from utilisation of an alternative

splice site in exon 9, and "Extremely short" (Es) which skips the exon 3 donor splice site thus converting exon 3 into an alternative terminal exon (Chubb et al., 2008). A combination of PCR on mRNA extracted from human hippocampus and 3' and 5' RACE identified up to 54 different isoforms with a varying expression profile. (Nakata et al., 2009). PCR with a series of primers produced five unpredicted products. These fragments were sequenced to reveal two products skipping exon 3 and exons 7 and 8, and three that end with an alternate exon 3. 5' RACE revealed products with first exons corresponding to the reported exon 1 of DISC1 or exon 1 of the adjacent Translin-associated factor X gene (TSNAX). 3' race gave rise to products with ten alternate final exons (Nakata et al., 2009). Not all of these variants are predicted to result in protein expression, as many contain a premature stop codon.

1.2.4 DISC1 Protein structure

The initial predicted structure for DISC1 was consistent with the hypothesis that it acts as a scaffold protein, having a globular head domain and an α-helical tail domain with numerous coiled coil protein binding sites and localisation signals (Millar et al., 2000). Further multiple sequence alignment work shows that the C terminus is more conserved throughout species than the N terminus region (Taylor et al., 2003). Despite this lack of conservation the N terminus domain is predicted to contain a nuclear localisation signal and an alpha helix. The remainder of the N terminus has multiple areas of low sequence complexity, or "disorder", which display a lack of folded structure with high flexibility (Figure 1.1, taken from Chubb *et al*, 2008). Contrary to original beliefs on the subject of disorder, this characteristic may actually be intrinsic to the function of the protein, allowing it to adopt a more ordered conformation on binding of further proteins (Tompa, 2002). The C-terminus of DISC1 also contains several self association domains, allowing for the formation of stable dimers and more labile oligomers (Leliveld et al., 2009).

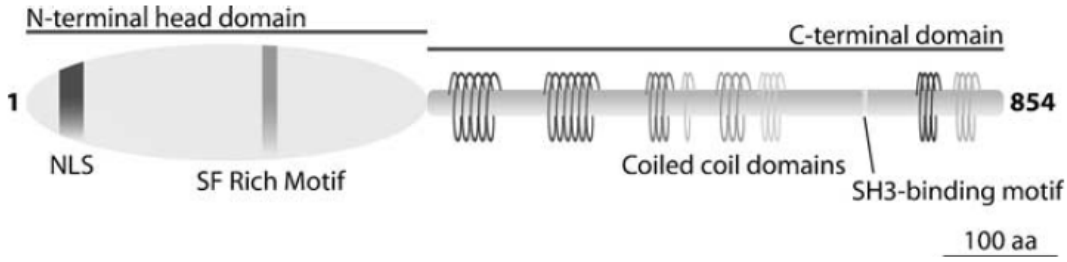


Figure 1.1 is taken from Chubb *et al* 2008, and is a simple summary of the structure of DISC1. The largely disordered N-terminal head domain contains a nuclear localisation signal, while the C terminal consists of a series of coiled coil domains implicated in protein binding and self association.

1.2.5 DISC1 Tissue Expression

DISC1 expression is not confined to the brain. Detailed discussion of mRNA and protein localisation in human and non human tissue can be found in Chubb *et al* 2008, thus this brief overview summarises mostly studies on human tissue. Northern blots demonstrate strong DISC1 mRNA expression in adult heart, widespread brain regions, placenta, and weak expression in the kidney and pancreas (Millar et al. 2000). RT-PCR demonstrates the expression of DISC1 in human foetal brain (Millar et al., 2000, James et al., 2004). Further work at the protein level confirms the presence of DISC1 in foetal brain, heart, limbs, kidney and lung (James et al., 2004).

RNA-based studies demonstrate strong expression of DISC1 transcripts in numerous human brain regions, including the amygdala, caudate nucleus, corpus callosum, hippocampus, substantia nigra, sub thalamic nucleus, thalamus, cerebellum, cerebral cortex, medulla, spinal cord, occipital pole, frontal and temporal lobes and the putamen (Millar et al., 2000). Despite the widespread expression of DISC1 with regards to brain regions, expression appears to be restricted to specific cell types within these areas (James et al., 2004).

In mouse studies, robust expression of DISC1 mRNA and protein was found throughout the developing hippocampus from E14, the first stage at which the hippocampus becomes discernable (Meyer and Morris, 2008, Austin et al., 2004). At E18 and P1 there is high DISC1 protein expression in the developing dentate gyrus and granule cell precursors. By P5 and P14 there is a well defined pattern of expression in Ammon's Horn and the dentate gyrus, one of the regions where adult neurogenesis occurs. In this neurogenic niche, DISC1 colocalises with Ki67, a marker of proliferation in the cells of the subgranular zone (Meyer and Morris, 2008). DISC1 is also robustly expressed in the other area responsible for adult neurogenesis, the subventricular zone, where it colocalises with neural progenitors expressing nestin and Sox2 (Mao et al., 2009). In the adult human, strong expression of DISC1 mRNA is observed in a subset of granule cells within the dentate gyrus, and weaker expression seen in hippocampal pyramidal cells in layers CA1-3 (James et al. 2004), both areas consistently associated with schizophrenia (Weinberger, 1999). Thus, DISC1 is highly expressed in the hippocampus, one of two areas of the brain that continues to undergo neurogenesis in the adult brain. Neurogenesis has been strongly implicated in psychiatric disease, a topic which I shall briefly discuss later in this introduction.

1.2.6 DISC1 developmental expression

Quantitative western blotting of mouse cortical lysate shows abundant presence of a 75kDa isoform of DISC1 throughout development. A 75kDa isoform shows low expression neonatally, but expression is upregulated at P25 and remains high until the final timepoint tested (6 months). A 105kDa isoform has continually low expression, whereas a 100kDa form has two peaks at E13.5 and P16.5. Expression of the 100kDa form also remains high after this second peak (Schurov et al., 2004). Interestingly, these peaks correspond to the occurrence of neurogenesis and neural migration and puberty in the mouse, both thought to be critical timepoints in schizophrenia pathogenesis (Lewis and Levitt, 2002). This work in mouse broadly concurs with similar studies in rat (Ozeki et al., 2003). In human dentate gyrus, prefrontal cortex and temporal cortex, a

riboprobe detecting exons 2-10 of DISC1 (and hence not the short forms) showed significantly increased expression from neonatal development to infancy, a marked decrease in expression by teenage years, and a more gradual decline of expression with age (Lipska et al., 2006). In humans therefore, the postnatal increase in DISC1 expression seems to come earlier than the corresponding timepoints in mouse: infancy versus puberty.

The more recent work describing the 54 putative splice variants of DISC1 also suggests differential regulation of DISC1 mRNA transcripts throughout human development, as seen in murine studies of protein. The transcripts skipping exons 3, 7 and 8 and the extra short variant are expressed more highly in the pre frontal cortex during gestation, and drop to continuously low levels throughout postnatal development and ageing (Nakata et al., 2009). Expression of longer transcripts follows a similar pattern, but the decline in expression is less severe. No peak in expression was seen postnatally. There is an effect of genotype at the intronic SNP, rs821597, on expression of short transcripts, where A/A homozygotes showed no decrease in short transcript expression with age. A similar but non significant trend was observed with variation at Ser704Cys (Nakata et al., 2009). Although there are some inconsistencies in this work, it is evident that isoform specific temporal regulation of DISC1 occurs throughout development. Further reconciliation of mRNA splice variants with putative protein products and their migration on gels will be required to reconcile these profiles into a complete developmental picture. DISC1 is therefore expressed throughout the brain during key events in development, neural migration, neurogenesis and synaptic pruning in adolescence (Meador-Woodruff and Healy, 2000).

1.2.7 DISC1 subcellular localisation

Light microscopy on post mortem human cortical samples shows prominent multi layer staining of pyramidal and non pyramidal neuronal cell bodies throughout the grey matter. This pattern does not appear to vary specifically between the four Brodmann areas studied. Electron microscopy shows frequent localisation of DISC1 to chromatin and ribosomes (Kirkpatrick et al., 2006). Unlike other studies (James et al., 2004, Ozeki et al., 2003, Millar et al., 2005a, Brandon et al., 2005), this one shows no localisation of DISC1 to the mitochondria. This difference may be important to note, Kirkpatrick *et al*, 2006 uses an antibody which is not employed in further publications. Light microscopy shows diffuse staining of the neuropil in both grey and white matter in these brain regions. Visualisation of these areas by electron microscopy localises the majority of DISC1 to synaptic structures including the axon terminals, the post synaptic density and dendritic spines. Staining is heterogenous, with only a proportion of the aforementioned structures expressing DISC1 (Kirkpatrick et al., 2006). DISC1, PDE4B, and their interactors NDEL1 and LIS1 are all present in synapse related cellular fractions (Clapcote et al., 2007, Niethammer et al., 2000), and exogenous protein colocalises to some extent with PSD95 in primary hippocampal neurons (Bradshaw et al., 2008). Further fractionation experiments demonstrate the presence of a 200kDa DISC1 immunoreactive protein in the core post synaptic density fraction, while a 75kDa band was found in large quantities in the synaptosome and vesicle enriched fraction (Bradshaw et al., 2008). It is generally accepted that at least a proportion of the pathophysiology of schizophrenia arises from dysfunction of the synapse (Harrison et al., 2005).

Subcellular expression of DISC1 protein varies between the cell types studied, and the antibodies used. Generally, DISC1 is predominantly cytoplasmic, and colocalises with markers for the mitochondria, cytoskeleton and centrosome. The major studies of DISC1 subcellular localisation in other cell types have been reviewed in great detail by Chubb *et al*, 2008. In primary cortical neurons, there is endogenous centrosomal localisation of DISC1 (Kamiya et al., 2005). In fact, DISC1 has been implicated in the

maintenance of the NUDEL/LIS1/Dynein complex at the centrosome, playing an important role in neural migration (Kamiya et al., 2005). Endogenous DISC1 is also present in a perinuclear distribution in primary cortical neurons (Brandon et al., 2005), and distributed in a punctate fashion with significant colocalisation with the mitochondrial marker HSP70. Overlap of expression with microtubular markers (MAP2 and α-tubulin) and disturbance of mitochondrial localisation post depolymerisation treatment shows association of DISC1 with microtubules in mouse cortical neurons (Brandon et al., 2005), and in rat hippocampal neurons (Taya et al., 2007). DISC1 is part of a molecular complex with KIF5A, LIS1, 14-3-3ε and NUDEL localised in the centre of axonal growth cones in d.i.v.3 rat hippocampal neurons. Some signal occurs in the peripheral and transitional regions of the growth cone (Taya et al., 2007). Finally, DISC1 has a conserved nuclear localisation signal (Brandon et al., 2005, Ma et al., 2002), has been detected in P1 nuclear subcellular fractions from brain and neural derived cell lines (Sawamura et al., 2005, Ozeki et al., 2003, James et al., 2004) and visualised in the nucleus by immunocytochemistry in SHSY5Y cells (James et al., 2004). This multi-compartmentalisation of DISC within the cell suggests that it may perform different roles and bind different proteins in the different cellular components, and thus have roles in a number of cellular processes. DISC1 dysfunction therefore, is likely to have multiple consequences for cellular function.

1.3 DISC1 Biological Function

Considering that next to nothing was known about the protein product of the DISC1 gene when it was first cloned in the year 2000, the explosion of studies, including animal models, *in vitro* biochemistry and cell culture experiments has been immense. As it stands in January 2010, over 250 papers are brought up by searching for "DISC1" on Pubmed, and in 2009 just over 1 paper per week was published on the subject. It is therefore impossible to cover every aspect of DISC1 biology in this review. As such, in this review I will focus on the evidence suggesting the potential for DISC1 to be

involved in modulation of intracellular signalling, referring to reviews and key pieces of work that fall outside this remit that can be consulted for further information.

1.3.1 The DISC1 interactome

Acknowledgement of the putative DISC1 structure as a scaffold protein led to multiple screens of various methods for potential protein interactors (Morris et al., 2003, Camargo, 2006, Miyoshi et al., 2004, Brandon et al., 2004, Millar et al., 2003, Ozeki et al., 2003, Shinoda et al., 2007 Taya, 2007 #221). The majority of work has been done via yeast-2-hybrid screening, with Shinoda (Shinoda et al., 2007) and Taya (Taya et al., 2007) publishing results from affinity chromatography and mass spectroscopy. Several proteins have been replicated in two or more of these original screens, or followed up via co-localisation, co-immunoprecipitation and GST pulldowns. Notable amongst these replicated results are PDE4B, ATF4 and 5, KIAA1212 (also named Girdin, AKT phosphorylation enhancer) LIS1, NDEL1 and NDE1, dynactin, SMARCE1, AKAP9, 14-4-3ε, Grb2, Spectrin, MIP-T3, SPTAN1 and EIF3. The reported interactors fall into 8 main gene ontology functional categories, namely; cell cycle regulation, RNA processing, transcription regulation, inter membrane and vesicle transport, cytoskeletal and membrane integrity, microtubule movement, and pertinently for this work, signal transduction and neural migration. Since these protein binding screens, further functional investigation of DISC1 has revealed a series of other interactors, the most interesting in this respect being glycogen synthase kinase 3β (GSK3β)(Mao et al., 2009).

These screens have raised an infinite number of future directions for research into the functions and potential role in the pathophysiology of schizophrenia of DISC1. The remit of this project being to investigate the potential for DISC1 to be involved in intracellular signalling, I will focus two of the interactors already known to mediate such pathways: the phosphodiesterase-4 protein family and GSK3β.

1.3.2 The Phosphodiesterase-4 family as DISC1 interactors

Yeast-2-hybrid screening with full length DISC1 demonstrates an interaction with PDE4B (Camargo, 2006), one member of a large family of 20 PDEs (Smith and Scott, 2006) which form the sole means of inactivating cAMP within the cell (Duman, 2002, Alberini et al., 1995). Local sculpting, both spatially and temporally, of cAMP gradients is crucial for normal cellular function, having implications for downstream enzyme activation and gene expression (Houslay, 2010). PDE4 is also the target of the prototypical antidepressant Rolipram (Dyke and Montana, 2002), and its significance in psychiatric disease pathophysiology will be covered in detail later in this introduction. PDE4 isoforms have the same basic structure, but contain unique N-terminal domains responsible for specific targeting to cellular locations, as detailed in Figure 1.2 (Houslay and Adams, 2003). Conventional and confocal microscopy in SHSY5Y cells and rat primary hippocampal cells shows colocalisation of endogenous PDE4B and the 71kDa isoform of DISC1 (Millar et al., 2005b). Exogenous FLAG tagged PDE4B colocalises with myc tagged DISC1 in dendritic spine structures when expressed in mature mouse neurons (Bradshaw et al., 2008).

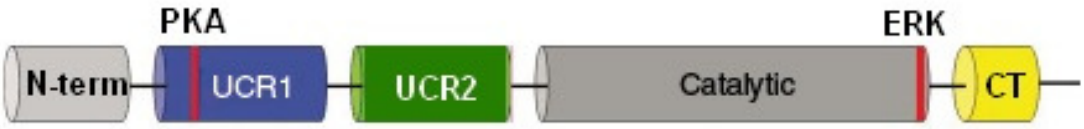


Figure 1.2, adapted from Burgin *et al*, 2010, shows the structure of a PDE4 long form. Long forms of PDE4 consist of a unique N-terminus region responsible for cellular targeting, two upstream conserved regions (UCRs) which act to modulate access to the catalytic site, a catalytic domain and C-terminus. Short forms may lack one of the UCR domains. PDE4 long forms have both a PKA and an ERK phosphorylation site, marked on this diagram with a red line.

Co-immune precipitation experiments strengthen the evidence for an interaction between the PDE4s and DISC1, but have also complicated the matter. All 4 human isoforms of PDE4 (A-D) precipitate with exogenous 100kDa full length DISC1 in HEK293 cells. On cAMP elevating treatment with IBMX (non specific PDE inhibitor) and forskolin (stimulates adenylate cyclase), PDE4C2 and PDE4D3 show a marked decrease in interaction with full length DISC1, releasing a PKA phosphorylated active PDE4 enzyme (Murdoch et al., 2007). This is in contrast to previous work on endogenous 71kDa DISC1, where PKA phosphorylated active PDE4B is released from DISC1 upon the same treatment of SHSY5Y cells (Millar et al., 2005b). Further investigation shows no release of long or short PDE4B isoforms from full length DISC1 upon cAMP elevation in HEK293 and SHSY5Y cells (Murdoch et al., 2007). This clearly has implications for compartmentalised and tightly regulated signalling, as the presence of certain isoforms of DISC1 and PDE4 in varying cellular locations will have different outcomes for PDE4 activity.

Early interaction experiments with deletion constructs implicated the N terminal head region of DISC1 and the UCR2 domain of PDE4B, as being sufficient for DISC1 binding (Millar et al., 2005b). Peptide array binding assays of 25mers of overlapping full length DISC1 sequence probed with MBP-PDE4B1 and MBP-PDE4D3 show 2 binding sites common to both probes, corresponding to amino acids 190-230 and 611-650. The first probe region is within that previously identified by deletion constructs. The PDE4B1 and a further PDE4B2 probe interacts with three additional regions of DISC1, residues 31-65, 101-135 and 266-290 (Murdoch et al., 2007). These results are confirmed in vitro using pull down of GST fusion proteins. Probing of DISC1 arrays with regions of PDE4D3 led to confirmation of the UCR2 domain as the first of the common binding sites. The catalytic unit of PDE4D3 identifies the second common site. Reciprocal arrays of PDE4D3 and PDE4B1 confirm these results as common DISC1 binding sites between B and D isoforms, and, intriguingly, found an additional specific binding site in the catalytic site of both PDE4B1 and PDE4D3. If the PDE4B specific

DISC1 site is removed through N terminal truncation of PDE4B, cAMP dependant release from DISC1 occurs (Murdoch et al., 2007).

An early model was proposed where DISC1 binds an inactive form of PDE4. An increase in intracellular cAMP activates PKA, which phosphorylates PDE4, resulting in its release in an active form from DISC1 (Millar et al., 2005b). Of course, this model is now complicated by the fact that this cAMP dependant release appears to be isoform specific (Murdoch et al., 2007). Recent work has attempted to solve the crystal structure of PDE4B and PDE4D when bound to the PDE inhibitors RS25344 and PMNPQ. The catalytic site of PDE4D7 contains a helical structure with a phenylalanine residue protruding from UCR2 into the active site. In PDE4B the corresponding residue is a tyrosine. This residue interacts with the inhibitors, suggesting an excellent target for subtype specific PDE4 inhibitors. The position of the UCR2 domain appears to be vital for PDE4 activity changes: antibodies targeting UCR2 can inhibit PDE4 activity alone. There are multiple residues in the UCR2 domain capable of interacting with the groove of the catalytic site. When these residues are mutated the affinity of the two inhibitors for PDE4 is decreased. Full and partial PDE4 inhibitors seem to act by "closing" the UCR2 domain across the active site of PDE4 (Burgin et al., 2010). DISC1 interacts with the UCR2 domain of PDE4B and PDE4D, and the catalytic domain of PDE4D (Murdoch et al., 2007). It is thus possible that DISC1 acts to inhibit PDE4s in a similar fashion to pharmacological inhibitors, and that PKA phosphorylation is capable of disrupting this interaction. This model does not however explain the varying susceptibility to release observed in biochemical experiments.

1.3.3 GSK3β as a DISC1 interactor

Unlike the previous three proteins, GSK3β was revealed as a DISC1 interactor by functional studies, not interaction screens. Knowledge that DISC1 expression peaks at E14-E15 in mice (Schurov et al., 2004), a period which coincides with neurogenesis, led to investigations of a putative role for DISC1 in cell proliferation. DISC1 knockdown

decreases, and overexpression increases proliferation of hippocampal progenitor cells both *in vitro* and *in vivo*. These effects are mediated via β-catenin and the Wnt signalling pathway, one of the major targets of the enzyme GSK3β (Mao et al., 2009). Indeed, DISC1 knockdown results in an increase in the activating autophosphorylation of GSK3β, while overexpression has the opposite effect. GSK3β coimmunoprecipitates with DISC1 from E14 mouse brain lysate, and binds directly to fragments 1-120 and 356-595 of DISC1 in a GST pulldown (Mao et al., 2009). The first DISC1 fragment, when mixed with purified GSK3β, β-catenin and axin (2 substrates), potently inhibits GSK3β autophosphorylation and downstream phosphorylation of β-catenin and axin. Fragments 221-355 and 356-595 have the same effect, but only at higher concentrations. In fact, a smaller peptide encompassing the highly conserved DISC1 residues 195-238 inhibits GSK3β autophosphorylation more potently than the commercial GSK3β peptide inhibitor L803-mts (Mao et al., 2009). Thus, not only does DISC1 appear to bind GSK3β, but it also has functional effects on the activity level of the enzyme, suggesting implications for downstream signalling of GSK3β mediated pathways. Indeed, DISC1 knockdown decreased the proliferation rate in adult hippocampal progenitor cells *in vitro* and *in vivo*. This effect was mediated via stabilisation of β-catenin, and rescued by overexpression of human DISC1 cDNA or pharmacological inhibition of GSK3β. Overexpression of DISC1 led to increased cell proliferation, again both *in vivo* and *in vitro* (Mao et al., 2009).

1.4 Animal models of DISC1 function

The validity of mouse models for such a uniquely human disease as schizophrenia has been discussed *ad nauseum* – how is it possible to model behaviour so intertwined with human senses and verbal communication in an animal? Although this is presently addressed in animal studies using very well characterised behavioural paradigms, with a large battery of controls for sensory deficits and confounding emotive behaviour, there remain questions over the validity of directly relating mouse behaviour to components of

schizophrenia. The fact remains that however one chooses to model the behavioural aspects of the disease, rodent models are hugely valuable tools as they allow genetic manipulation, developmental studies, investigation of gene environment interactions and pharmacological interventions which are considered unethical in human subjects. Whilst the biological processes occurring in the rodent models may not be identical to those of humans, with regards to the DISC1 pathway the expression pattern of DISC1 is relatively well conserved, despite the relatively low sequence conservation between humans and rodents (Bord et al., 2006). Conventional knock outs of DISC1 have proved difficult to produce, possible due to the transcriptional complexity of the DISC1 gene, and so various alternative approaches have been taken to assess DISC1 biology. Once more, it is outside the scope of this review to cover every animal model involving DISC1, so I shall focus on those that show specific relevance to alterations in intracellular signalling pathways.

1.4.1 DISC1 murine models

Many groups have genetically altered DISC1 expression in mice, to assess the phenotypes resulting for these changes. In this section I will attempt to write a concise summary of the other mouse models that have addressed the DISC1 phenotype, and allude towards possible signalling pathways that may be affected. It has been reported that the 129s6/SvEv strain of mice harbour a naturally occurring deletion in DISC1, and this variant has been expressed on a C57BL/6 background to assess potential behavioural effects (Koike et al., 2006, Kvajo et al., 2008). The two main avenues of investigation however, have been to over express truncated forms of DISC1 to mimic the potential protein product from the translocation family (though it is important to state that no biological evidence of this protein has been discovered in the family, simply a 50% reduction of DISC1 protein, Millar et al., 2005b), and to knock down DISC1 using shRNA. In essence, both approaches model a decrease in DISC1 expression, as the truncated product generally has a dominant negative effect on wild-type DISC1 localisation through self association, and thus results in a decrease in DISC1 expression

at its normal locations. A final investigation took the alternate approach of applying serial electrical stimuli to the rat amygdala in an attempt to model the effects of seizures. In this case DISC1 expression was tested retrospectively, and found it to be decreased in granule cells of the dentate gyrus (Fournier et al., 2009).

A huge array of DISC1 phenotypes are reported in these animal models, but there are common themes that occur in many of the experiments. These are summarised in Table 1.1A and B. The reported phenotypes include large scale architectural changes, such as enlargement or abnormal asymmetry of the lateral ventricles during at least one stage of mouse development (Shen et al., 2008, Hikida et al., 2007, Pletnikov et al., 2007, Ayhan et al., 2010), a phenomenon also reported in schizophrenic humans (McCarley et al., 1999). Three experiments report a decrease in neural progenitor proliferation, which Mao *et al* describe as a function of aberrant GSK3β and Wnt signalling (Kvajo et al., 2008, Shen et al., 2008, Mao et al., 2009). Abnormal neural migration and ectopic integration is a feature of most models (Kamiya et al., 2005, Kvajo et al., 2008, Duan et al., 2007, Meyer and Morris, 2009). One experiment suggests that only the functionally diverse granule cells in the dentate gyrus, and not the excitatory pyramidal cells are affected by this phenotype (Meyer and Morris, 2009) One experiment attempted to further characterise which extracellular signalling pathways may be affected by overexpression of truncated DISC1 by assessing levels of neurotransmitters and their metabolites. There is a decrease in dopamine and the dopamine metabolite 3,4-dihydroxyphenylaceticacid (DOPAC) in the frontal cortex of male mice expressing truncated DISC1, and a decrease in dopamine in the hippocampi of female mice with truncated DISC1 expressed post natally (Ayhan et al., 2010). The dopamine hypothesis of schizophrenia, which is partly formulated on the concept of dopamine hypofunction in the pre frontal cortex, will be discussed in detail later in this introduction.

Experiments on brain slices from DISC1 knockdown mice suggests an increased rate of synapse maturation on dendritic spines, (Duan et al., 2007), while one experiment showed decreased synaptic transmission but with intact long term potentiation (LTP)(Li

et al., 2007a). Overexpression of truncated DISC1 results in a decrease in dendritic complexity (Pletnikov et al., 2007, Shen et al., 2008, Li et al., 2007a), while there is a variable effect on dendritic spine density (Kvajo et al., 2008, Ayhan et al., 2010, Shen et al., 2008, Fournier et al., 2009). Dendritic complexity is thought to be a result of expression of specific combinations of transcription factors and actin remodelling in response to integration of a number of extracellular and intracellular signals (Gao, 2007, Tada and Sheng, 2006). It is thus possible that DISC1 dependent changes in the activity of GSK3β or the PDE4s may lead to differential transcription from LEF/TCF and CREB binding elements, to alter dendritic complexity. DISC1 also directly binds transcription factors (Camargo, 2006), and may act as a scaffold for assembly of transcriptional complexes and actin associated proteins (Tada and Sheng, 2006). Indeed, transcriptional assays show an additive effect of DISC1 and ATF4 expression in CRE-driven luciferase assays, suggesting that DISC1 is responsible for recruiting transcriptional repressors to ATF4 containing transcriptional machinery. ATF4 has been implicated in multiple brain functions, including a form of long term memory, as one of the CRE binding family of CREB transcription factors. (Sawamura et al., 2008).

Finally, behavioural tests that recapitulate some of the features of psychiatric disease including depression (forced swim and tail suspension tests), hyperactivity (novelty induced locomotion), cognitive and sociability deficits were performed, and all show positive results in some experiments. Table 1.1A and B summarise the results of these tests. None of these models showed changes in anxiety related behaviour. As can be seen from the table, three investigations report locomotor hyperactivity in the open field, while 3 report no effect on this paradigm. Working memory is compromised in two models (Kvajo et al., 2008, Koike et al., 2006, Clapcote et al., 2007). There are however, almost as many negative findings throughout these experiments as there are positive, the majority of papers failing to replicate an aspect of behaviour that other groups report. There may be multiple reasons for this. Although the protocols for these tests are relatively standard, the conditions, equipment and levels of attention the mice are kept in will all vary between institutions. The exact expression pattern of DISC1,

both temporally and spatially, may well result in different behavioural phenotypes. Two of the groups have already shown that the timing of DISC1 overexpression can significantly alter the magnitude of the phenotype observed (Ayhan et al., 2010, Li et al., 2007a). What is apparent is that changes to expression levels of DISC1 result in an array of behavioural phenotypes in mice, all of which can be related to aspects of human psychiatric disease. This would seem to add evidence to the idea that specific psychiatric diseases are less defined than originally perceived, and that DISC1 may well act as a hub protein, whose specific mutation, presence or absence can leave one susceptible to a range of psychiatric conditions.

1.4.2 DISC1 point mutations lead to "schizophrenic" and "depressive" like behaviour – a role for DISC1/PDE4 interactions?

Clapcote *et al* took the approach of screening a bank of ENU mutagenised sperm for missense mutations in the largest coding Disc1 exon, exon 2, and found two independently derived missense mutations, Q31L and L100P. Although there are no genotype related differences in mouse viability, two phenotypes with central shared features are apparent from in depth testing of these animals. Both homozygous mutants have decreased brain volumes in comparison to wild type mice, despite expressing normal levels of all measured isoforms of DISC1. Both have decreased Prepulse Inhibition (PPI, a measure of information processing deficits, commonly subnormal in schizophrenic patients, Gottesman and Gould, 2003) in comparison to wild type litter mates. (Clapcote et al., 2007). This deficit is more pronounced in L100P animals, and partially alleviated by the common antipsychotics haloperidol and clozapine. Interestingly, the deficit is abolished by rolipram in the L100P mouse and the antidepressant bupropion in Q31L animals. Latent Inhibition, a measure of information processing when mice are pre exposed to a conditional stimuli, is also disrupted in both homozygous mutants and L100P heterozygotes, and abolished by clozapine in the L100P homozygotes. Both Q31L and L100P heterozygotes show working memory deficits (Clapcote et al., 2007).

L100P mice display increased horizontal locomotion in the open field test which is not related to elevated anxiety. This leads to a suggestion of "schizophrenic" type behaviour in L100P mice. Q31L homozygous mutants display a quite different phenotype, including increased immobile floating time in the Forced Swim Test. Both bupropion and rolipram decrease immobile time in wild type rats, but only bupropion was effective in Q31L mutants. Q31L mutants also exhibit decreased social interactions and lower reward responsiveness. This leads to the suggestion that these mice are behaving in a "depressive" like fashion, potentially due to the effect of the mutation on PDE4B binding to DISC1 Both DISC1 mutations fall within regions implicated in PDE4B specific binding via peptide array (Murdoch et al., 2007). Indeed, in vitro experiments demonstrate decreased binding of PDE4B to both mutant forms of DISC1, with the L100P mutation having the greatest effect. Brains from Q31L homozygote animals also show a marked 50% decrease in PDE activity, despite their possessing an equal amount of protein to their wild type littermates. This most likely explains their lack of sensitivity to rolipram treatment (Clapcote et al., 2007). This exciting work clearly demonstrates how small structural changes in DISC1 can have a large impact, not only on the biochemistry of DISC1-PDE4 interactions, but that these changes are significant enough to be detected in behavioural paradigms, which respond differentially to drug treatment. This implicates DISC1 and PDE4 in behaviour that relates to two different human psychiatric conditions, and increasingly suggests a role for both allelic heterogeneity of DISC1 and altered DISC1/PDE4 interactions in psychiatric conditions.

A

Phenotype	Clapcote 2007 ENU mutant Q31L	Clapcote 2007 ENU mutant L100P	Mao 2009 FL human DISC1	Koike 2006 Truncated DISC1, endog. promoter	Kvajo 2008 129	Shen 2008 Truncated mouse BAC	Kamiya 2005 Truncated DISC1 with exogenous promoter	Hikida 2007	Li 2007 P7	Pletnikov 2008 Truncated inducible DISC1 with exogenous promoter E15	Ayhan 2010 P. & P.	Ayhan 2010 Pre	Ayhan 2010 Post
Brain volume	↓	↓		NS		↓ male		NS		NS	NS	↓	NS
Lateral ventricles						↑		↑		↑	↑	NS	NS
Cortical volume	↓	↓		NS	↓ PFC	↓		NS		NS	↓	NS	↓
Neural proliferation			↑		↓	↓							
Neurite outgrowth					↓	↓				↓			
Parvalbumin in cortex					NS	↓		↓			↓	↓	↓
Open field locomotion	NS	↑	NS	NS		NS		↑	NS	↑ male			
Aggressive behaviour										↑ male	↑ m.	NS	↑ m.
Sociability	↓	NS						NS	↓	↓ male			
Spatial learning/memory	NS	NS			NS			NS		↓ female			
Working memory	↓	NS		↓	↓				↓				
Prepulse inhibition	↓	↓		NS				↓		NS			
Latent inhibition	↓	↓				↓							
FST immobile time	↑	NS	NS			↑		↑	↑		NS	NS	↑ f.
Dendritic complexity					NS	↓			↓	↓			
Ectopic integration					↑	↑	↑						
Dendritic spine density					↓						↑	↑	NS
Dopamine											↓ DA PFC m., HP f.		

B	Kamiya 2005	Duan 2007	Mao 2009	Meyer 2009
Neural proliferation			↓	
Open field locomotion			↑	
FST immobile time			↑	
Dendritic complexity		↑ granular cells	↑	
Neural migration	Defect	Defect		Defect
Ectopic integration				↑
Dendritic spine maturation		↑		
Synapse maturation		↑		

Table 1.1A, adapted from Shen *et al*, 2008, on the previous page details the phenotypes observed in various mouse models of DISC1 function, including ENU mutant DISC1, overexpression of human full length DISC1, expression of truncated mouse DISC1 variants, and expression of truncated DISC1 to model the human translocation. ↓ = reduced, ↑ = increased, NS = no statistically significant effect, and a blank box means this investigation was not reported on. Abbreviations used are: P. & P. = pre and post natal expression of inducible DISC1 construct, m. = male, f.= female, HP = hippocampus. Table 1.1B details the phenotypes observed in shRNA DISC1 knockdown experiments.

1.4.3 Non Mammalian DISC1 models

The transgenic approach of DISC1 study has recently been extended to non mammalian animals, including zebrafish and drosphila, despite there being no DISC1 fly orthologue (Bord et al., 2006). Transgenic flies express full length human DISC1 in mushroom bodies, which are structures involved in cognition, associative learning, attention and sleep homeostasis. Expression of full length human DISC1 causes a mild sleep related phenotype, where male flies spent increased time asleep per day (Sawamura et al., 2008). Disturbances such as these have been related to cAMP Response-element binding proteins (CREB), transcription factors which bind to cAMP responsive elements (CRE) (Hendricks et al., 2001), with evidence from non-mammalian experiments investigating PDE4 orthologues suggesting high cAMP leads to decreased sleep (Zimmerman et al., 2008, Drerup et al., 2009).

Strikingly, DISC1 morphant zebrafish portrayed a similar phenotype to those of another schizophrenia susceptibility gene: neuregulin. DISC1 morpholino-injected (an anti-sense method of knock-down) embryos have numerous gross developmental abnormalities: failed swim bladder inflation, mis-shapen eyes and head, cartilaginous and pectoral fin abnormalities, oedema in the pericardium and possessed no articulated lower jaw. Most of these changes are likely attributed to the absence of DISC1 in migrating cranial neural crest cells, which are reduced in the pharyngeal arches of morphants (Wood et al., 2008, Drerup et al., 2009). Morphants express a dose dependent decreased population of oligodendrocyte precursor olig2 expressing cells, and more terminally differentiated Sox10 expressing cells in the hindbrain. This change was specific to DISC1 knockdown, and not attributable to p53 induced apoptosis (Wood et al., 2008). This is interesting as oligodendrocyte abnormalities, including decreased total cell counts (Hof et al., 2002), and cytological changes (Uranova et al., 2001) have been observed in schizophrenic post mortem tissue, and white matter integrity changes are commonly reported throughout psychiatric disease (White et al., 2008). However, evidence from mouse models has failed to show such phenotypes in response to DISC1

manipulation – could it be that mammals have evolved greater redundancy in pathways responsible for myelination, due to the greater distance that neurons must migrate?

1.5 DISC1 affects signalling pathways associated with major psychiatric disease

As with all aspects of DISC1 research, there has been an almost exponential increase in *in vitro* studies of DISC1 over the past 5 years. For this reason I will confine my introduction only to those studies that highlight effects of DISC1 on well established signal transduction pathways that have been associated with psychiatric disease, independent of their association with DISC1, including ERK1/2, AKT and GSK3β and cAMP. While these three proteins find themselves in pathways that are canonically viewed as isolated, there is a large amount of cross talk and interaction which has been highlighted as important in mood disorders and schizophrenia. Here I will focus on the pharmacological aspects of these associations.

1.5.1 Neurotrophin signalling and mood disorders

ERK1/2 is part of the ras-mitogen-activated protein kinase (MAPK) cascade, whose most well characterised role is to prevent cells from undergoing programmed cell death. AKT forms parts of the PI3K/AKT pathway, responsible for activating a multitude of downstream effectors, including AKT, in response to the formation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) from phosphatidylinositol 4,5-bisphosphate by PI3K. Both cascades are implicated in regulation of synaptic plasticity, formation of dendritic spines, and neuronal complexity, differentiation and survival by activation in response to neurotrophins such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Yuan et al., 2001, Riccio et al., 1999, Mai et al., 2002, Foulstone et al., 1999, Alonso et al., 2004, Atwal et al., 2000, Jaworski et al., 2005 Jiang, 2005 #544). BDNF is of particular interest in depression as mRNA is decreased in the hippocampus and dentate gyrus in rats undergoing chronic

immobilisation stress (Smith et al., 1995), an adaptation which is blocked by administration of antidepressants (Nibuya et al., 1995). When administered centrally BDNF itself has antidepressant effects in at least two behavioural tests: the forced swim test and learned helplessness post shock (Siuciak et al., 1997). Chronic administration of antidepressants increases BDNF mRNA in the hippocampus (Nibuya et al., 1995). Treatment of SHSY5Y cells with BDNF leads to a decrease in GSK3β activity via AKT mediated phosphorylation of Ser9 and results in increased phosphorylation of the cAMP response element binding protein (CREB), as does treatment of cells with lithium (Cross et al., 1995, Grimes and Jope, 2001a). Overexpression of GSK3β blocks BDNF induced phosphorylation of CREB, except in the presence of lithium, which also causes a small increase in ERK1/2 and CREB phosphorylation in control (non-BDNF treated) cells (Mai et al., 2002).

CREB is one of the major downstream effectors of neurotrophins, and has similar antidepressant effects to BDNF when expressed in the hippocampus (Chen AH et al., 2001). CREB is activated by chronic antidepressant treatment (Thome et al., 2000), ERK (via ribosomal S6 kinase) and PKA by phosphorylation at Ser133 (Mai et al., 2002, Coyle and Duman, 2003, Shaywitz and Greenberg, 1999) and negatively regulated by GSK3β at Ser 129 (Grimes and Jope, 2001b). ERK and AKT signalling is activated by phosphorylation after chronic treatment with valproate, and short term treatment with carbamazepine enhances basal and BDNF stimulated ERK1/2 and CREB phosphorylation (Yuan et al., 2001, Mai et al., 2002). Both of these drugs are commonly used mood stabilisers. Phosphorylation of AKT in response to valproate treatment is accompanied by an increase in inhibitory phosphorylation at Ser9 of GSK3β, while lithium treatment has this same effect without an increase in AKT phosphorylation (De Sarno et al., 2002). Chronic valproic acid treatment of SHSY5Y cells in serum free media leads to an ERK mediated increase in neurite formation and cell survival, and an increase in Bcl-2 protein (Yuan et al., 2001), whose promoter activity is regulated by CREB binding (Riccio et al., 1999).

This wealth of information leads to a central pathway inextricably linked to depression and bipolar disorder, by its outcomes (control of neuronal plasticity and survival), its modulation at various points by antidepressant and mood stabilising drugs, and by post mortem evidence from human disease. Schizophrenic brains have 68% decreased levels of AKT protein in peripheral tissues, and decreased levels in post-mortem frontal cortex and hippocampus when compared to a control population (Emamian et al., 2004a). There are concomitant decreases in GSK-3 phosphorylation at Ser9 in the periphery and brain post mortem tissue (Emamian et al., 2004a). Finally, post mortem frontal cortex shows an array of disturbances in the MAPK cascade with respect to psychiatric diagnoses, including a significant decrease in MEK1 (which acts upstream to activate ERK1/2) in bipolar disorder, major depression and schizophrenia, and decreased CREB in major depression and schizophrenia regardless of pre morbid use of medication. There were no changes in protein expression of ERK1/2, and unfortunately potential changes in phospho proteins as are observed in many of the animal models were not assessed (Yuan et al.).

To summarise (and grossly simplify!), growth factors, particularly BDNF, bind to tyrosine kinase receptors and activate PI3K and MAPK cascades, resulting in phosphorylation of ERK1/2 and AKT. Activation of ERK leads to activating phosphorylation of CREB, which binds to CRE response elements in multiple genes, resulting in transcription of neuronal plasticity and survival related genes including Bcl-2. Concomitant activation of AKT results in inhibition of GSK3β, removing the inhibitory effect of this enzyme on CREB. This basic pathway can be stimulated by mood stabilising and antidepressant drugs either by direct inhibition of GSK3β or upstream activation of ERK and AKT (Coyle and Duman, 2003). This pathway therefore has three major outcomes with implications for psychiatric disease: activity changes downstream of ERK, including CREB mediated gene transcription, activity changes downstream of AKT, including activation of mammalian target of rapamycin (mTOR) which phosphorylates eIF-4E binding protein to regulate translation (Jaworski

et al., 2005, for a comprehensive review see O'Donnell and Gould, 2007), and changes in GSK3β activity. This process is summarised and simplified in figure 1.3.

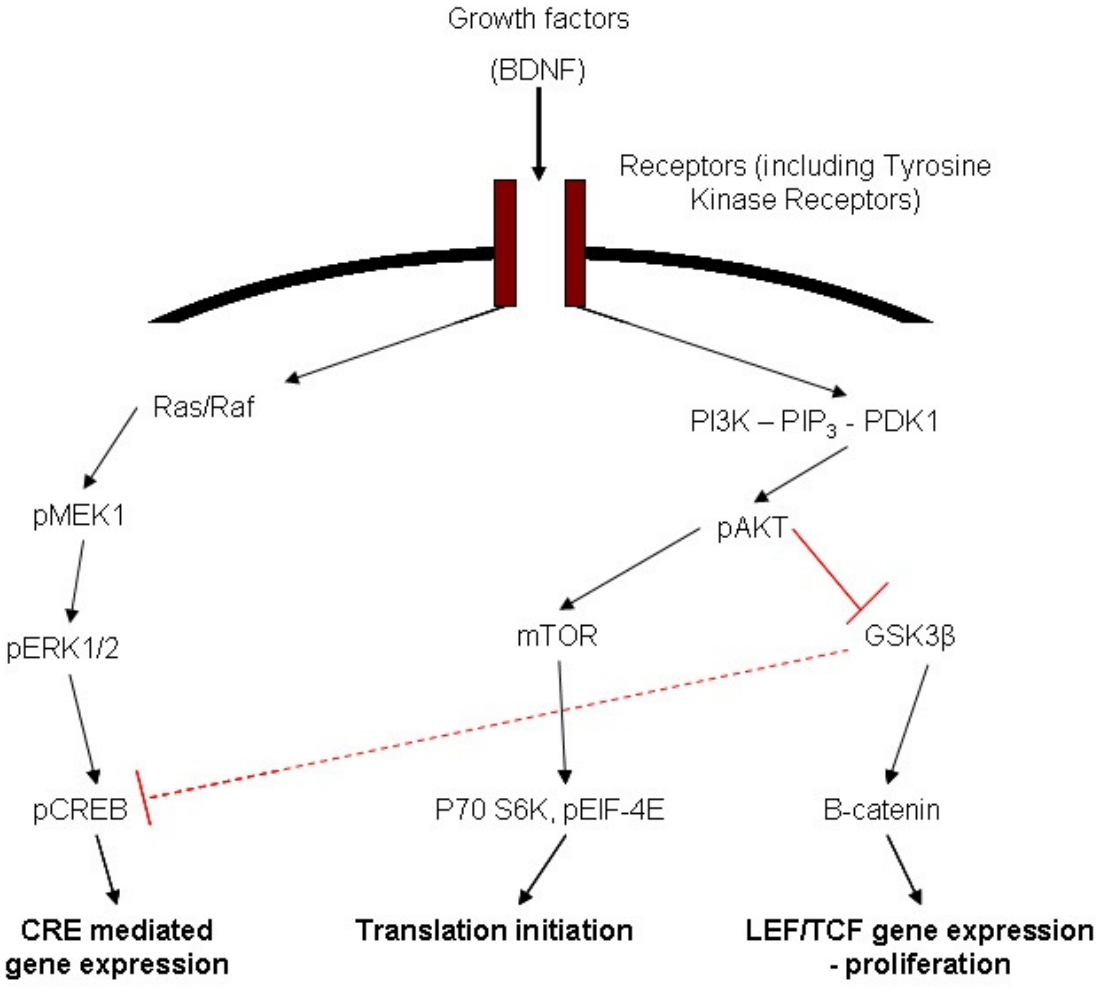


Figure 1.3 depicts a simplified version of a selection of the pathways involved in neurotrophin signalling, showing how signalling from growth factor receptors leads to three different means of regulation neuronal proliferation, growth and differentiation.

1.6 Lithium:mechanisms for mood stabilisation

Lithium remains the most established treatment for bipolar disorder. There have been a huge number of behavioural studies performed on wild type rodents to assess the effects of lithium treatment, which suggest antidepressant, anxiolytic, and anti-manic properties. Amongst the most well replicated of these experiments are decreased aggression, decreased amphetamine, reserpine and stress induced hyperactivity, decreased exploratory behaviour, decreased latency to enter light compartments, decreased immobile time in the forced swim test, and an increase in pilocarpine induced seizures on lithium treatment (Beaulieu et al., 2008, O'Donnell and Gould, 2007). It is still unclear whether there is a definitive molecular mechanism by which lithium exerts its effects, and at present there are two major pathways implicated: disruption of inositol homeostasis and inhibition of GSK3β. These two mechanisms are by no means mutually exclusive.

The inositol depletion hypothesis stemmed from the observation that exogenous inositol protected Xenopus embryos from lithium induced embryo malformation (Busa and Gimlich, 1989). It was later discovered that lithium inhibits the activity of two of the lipid phosphatase enzymes responsible for hydrolysing, and thus recycling, inositol-1,4,5 trisphosphate (IP3). This leads to an effective depletion of inositol which is particularly apparent in the brain, due to the lack of permeability to inositol of the blood brain barrier (reviewed Berridge et al., 1989). IP3 is a major second messenger downstream from GPCRs, and is involved in releasing calcium from intracellular stores to activate calcium regulated proteins. As with cAMP and PDE enzymes, which act to rapidly regulate cAMP fluxes both spatially and temporally, lipid phosphatases are required to inactivate this IP3 signal. More recent behavioural work in mammals is increasingly critical of this hypothesis as an important function in the efficacy of lithium (reviewed Beaulieu and Caron, 2008). The most striking of these investigations shows that mice with a mutated myo-inositol transporter, who have inositol depleted in the PFC and hippocampus to similar levels to those reported in humans and rodents after chronic lithium treatment (Beaulieu and Caron, 2008), fail to recapitulate any of the noted behavioural effects of

lithium treatment. These mutant mice were not sensitive to pilocarpine induced seizures, and showed no difference in the forced swim test and amphetamine induced hyperactivity when compared to wild type mice (Shaldubina et al., 2007).

GSK3β was originally proposed as a target of lithium after observations that the teratogenic effects lithium causes in Xenopus embryos were markedly similar to the effects resulting from disruption or inhibition of GSK3β and wingless (Wnt) signalling. Indeed, bacterially purified GSK3β was inhibited by lithium in vitro with an IC_{50} of 2mM, meaning incomplete inhibition will occur within the therapeutic ratio of 0.5-1.5mM lithium. In *in vitro* enzyme reaction studies however, LiCl became more potent at inhibiting GSK3β as Mg^{2+} concentration decreased. This implies that the clinical effect of lithium could vary between tissues, dependent on their magnesium content, with the potential for lithium to be more potent in vivo than in *in vitro* studies (Ryves and Harwood, 2001). This inhibition of GSK3β by lithium was shown to be uncompetitive (Klein and Melton, 1996). 2mM LiCl was ineffective against activity of other kinases tested, including JNK, MAPK and ERK 1 and 2. Activity of overexpressed GSK3β was then shown to be altered by lithium in COS1 cells, using phosphorylation of co-transfected Tau as a proxy for GSK3β activity. Finally, addition of lithium to S2 cells (cells of drosophila origin lacking the Frizzled receptor) and mammalian PC12 cells resulted in an accumulation of the drosophila β-catenin homologue Armadillo and β-catenin respectively. This demonstrated that Gsk3 and lithium function downstream of Wnt receptor signalling (Stambolic et al., 1996).

1.7 GSK3β: multiple pathways for psychiatric pathogenesis

As discussed above, GSK3β is an enzyme strongly implicated in psychiatric illness by virtue of its inhibition by LiCl, a common mood stabiliser (Beaulieu and Caron, 2008) and primary treatment for Bipolar Disorder. GSK3β is a virtually ubiquitous protein with a huge number of putative substrates, some of which are detailed below. Fine control of

this enzyme is therefore key to appropriate function, and can be achieved at a series of levels. The first level is phosphorylation. GSK3β is phosphorylated at the inhibitory Ser9 site by a number of proteins, including AKT and PKA (Brian et al., 1982, Cross et al., 1995). GSK3β also possesses an activating phosphorylation site, but there is controversy over which kinases may act here, and whether GSK3β may autophosphorylate (Jope and Johnson, 2004, Grimes and Jope, 2001b). The second level of GSK3β activity control is also related to phosphorylation. Although GSK3β does not require a strict canonical motif in a substrate, priming phosphorylation of the substrate by a separate kinase is often required for efficient phosphorylation (Fiol et al., 1987).

The third level of control lies in the presence of GSK3β in a larger complex. For example, in the canonical Wnt signalling pathway, binding of Axin to GSK3β enhances phosphorylation of β-catenin by GSK3β (Ikeda et al., 1998). In a completely separate complex, GSK3 binding protein (GBP) bind GSK3β to sterically inhibit the active site (Grimes and Jope, 2001b). There is some evidence that binding of these protein complexes may be competitive (Farr et al., 2000). This effect of protein complexes links with the fourth level of enzyme control: subcellular localisation. GSK3β contains a highly conserved nuclear localisation signal, yet its expression is predominantly cytoplasmic. However, the proportion of nuclear GSK3β increases during significant cellular events such as S phase of the cell cycle. When GSK3β–HA is overexpressed, there is a 50% increase in the nuclear:cytoplasmic ratio of GSK3β, suggesting the presence of cytoplasmic anchoring proteins that are saturated by overexpression of GSK3β. Indeed, if cells are chemically crosslinked, the majority of endogenous GSK3β can be found in complexes (Meares and Jope, 2007). Thus GSK3β activity may also be regulated by control of subcellular localisation, mediated by cytoplasmic proteins who anchor it in specific cellular compartments. These multiple levels of control allow for very specific GSK3β mediated actions from a largely ubiquitous enzyme.

GSK3β is expressed in many tissues throughout the body, but particularly enriched in brain (Woodgett, 1990). In the cortex, layers V and VI are the most immunoreactive,

and in the hippocampus the signal is strong in pyramidal neurons, while the granular neurons of the dentate gyrus are weakly labelled. Immunocytochemistry shows GSK3β to be present throughout hippocampal neurons, in dendritic spines, and it is found in both the cytosolic and synaptosomal fractions prepared from hippocampal and cortical neurons (Peineau et al., 2007, Claudie et al., 2007). Studies in rat brain suggest that though GSK3β expression is high throughout development, there is a peak of expression between embryonic day 18 and postnatal day 10, which corresponds to the major period of synapse formation and maturation (Leroy and Brion, 1999).

Pharmacological and genetic manipulation of GSK3β related pathways has strengthened the case that inhibition of GSK3β is vital for neuroactive components of lithium treatment. GSK3β heterozygous knock out mice recapitulate the behavioural effects of lithium treated wild types, demonstrating decreased immobile time in the forced swim test, decreased exploratory behaviour, decreased amphetamine induced hyperlocomotion (Beaulieu et al., 2004, Beaulieu and Caron, 2008, O'Brien et al., 2004). Lithium treatment of GSK3β heterozygous knock out mice results in impeded locomotion, while GSK3β homozygous knock outs are embryonic lethal (O'Brien et al., 2004), suggesting that there is an optimal level of GSK3β inhibition for beneficial neuroactive effects.

Pharmacological inhibition of GSK3β by specific inhibitors produces effects in mice very similar to those exhibited in response to lithium treatment. Thiadazolidinone (TDZD-8) inhibits novelty induced locomotor activity, and decreases anxiety as measured by the latency to enter a light compartment (Beaulieu et al., 2008, Gould et al., 2004). In mice with the dopamine transporter DAT knocked out, LiCl antagonises dopamine dependant hyperactivity, as do the specific GSK3β inhibitors SB216763, sodium valproate and TDZD-8 (Beaulieu et al., 2004). Finally, GSK3β specific inhibition also leads to decreased immobile time in the forced swim test (Kaidanovich-Beilin et al., 2004, Gould et al., 2004), one of the most well replicated effects of lithium treatment (O'Donnell and Gould, 2007).

Brain specific expression of a constitutively active form of GSK3β results in decreased food intake and brain size (whilst rates of hippocampal and sub ventricular proliferation are maintained), increased locomotor activity with decreased habituation to novelty, hyper-responsiveness to acoustic startle and, somewhat counter intuitively, decreased immobility in the forced swim test (Prickaerts et al., 2006). AKT expression is upregulated in the striatum of these mice, presumably to compensate for GSK3β over-activation. This hypothesis is strengthened by the presence of increased phosphorylation at Ser9 of endogenous GSK3β in the striatum of these mice (Prickaerts et al., 2006).

In cell culture and post-mortem studies, treatment with antipsychotic drugs has effects on GSK3β activation. Short term treatment of SHSY5Y cells with clozapine results in increased inhibitory phosphorylation of Ser9 of GSK3β, and hence an accumulation of β-catenin in the nucleus (Kang et al., 2004). Acute risperidone and olanzapine treatment increases GSK3β Ser9 phosphorylation in the cortex, hippocampi, striatum and cerebellum of mice, but has no effect of levels of phosphor-AKT. Clozapine has the same effect, but this is only significant in the cortex and hippocampus. Interestingly, lower doses of risperidone have a greater effect on GSK3β phosphorylation than high doses. In the same paradigm, haloperidol has no effect (Li et al., 2007b).

1.7.1 Downstream effectors of GSK3β

GSK3β has a large number of downstream effectors, the most well studied of which is β-catenin, a scaffolding protein involved in cell adhesion and major player in the Wnt signalling pathway critical for controlling cell proliferation. GSK3β inhibits the accumulation of β-catenin through phosphorylation leading to ubiquitination (Beaulieu and Caron, 2008), while DISC1 has been proven to stabilise β-catenin through an inhibitory effect on GSK3β (Mao et al., 2009). β-catenin is also implicated in dendritic morphogenesis in a pathway independent of downstream Wnt signalling, with overexpression resulting in more complex dendritic arborisation with shorter branching. N-cadherin mediated sequestration of β-catenin resulted in a decrease in axon branching (Yu and Malenka, 2003). This may go some way to explaining the changes in synaptic

complexity seen in DISC1 mutant mice. Finally, β-catenin is potentially involved in synaptic plasticity through NMDA receptor activity dependent redistribution to neuronal spines (Murase et al., 2002).

A large number of other substrates of GSK3β have been described and are summarised in Table 1 of an excellent review by Grimes and Jope (Grimes and Jope, 2001b). Amongst these is PKA (also known as cAMP dependent protein kinase) (Brian et al., 1982), which is known to phosphorylate, and hence activate PDE4 long isoforms (MacKenzie et al., 2002). Also of note, GSK3β phosphorylates numerous cytoskeletal and cell adhesion related proteins, suggesting it may play a crucial role in neuronal architecture, and a large collection of transcription factors, the key end points of many intracellular signalling pathways (Grimes and Jope, 2001b).

Finally, more recent work has implicated GSK3β in two processes critical for synaptic plasticity, NMDA dependent long term potentiation and depression. Induction of LTD resulted in an increase in GSK3β activity in CA1 of the hippocampus. GSK3β inhibitors prevented the induction of LTD in *in vivo* recordings from 2 week old rat hippocampi (Peineau et al., 2007). Conversely, LTP inhibits GSK3β activity in vivo and in vitro through inhibitory phosphorylation at Ser9 (Claudie et al., 2007, Peineau et al., 2007) while transgenic mice overexpressing GSK3β have significant deficits in LTP and spatial learning deficits that are rescued by lithium treatment (Claudie et al., 2007). Induction of LTP and LTD using electrical stimulation are common experimental paradigms used to model the chemical strengthening and weakening of synapses respectively. These processes are believed to provide information about similar processes which occur during learning and the formation of memory (Malenka and Bear, 2004). It is as yet uncertain exactly what the role of GSK3β is in this process, how essential it is, whether it acts as an integrator of higher signals (Wnt, for example), or whether it has a closer association with NMDA and AMPA receptors and the process of internalisation. Indeed, neither of these roles is mutually exclusive.

The evidence for GSK3β playing an important role in multiple psychiatric disorders is multi leveled and growing at an astounding rate. Pharmacological evidence that began with lithium treatment in humans has led to an increasing repertoire of substrates this ubiquitous enzyme phosphorylates, and links it to multiple processes implicated in psychiatric disease: synaptic plasticity, CREB signalling, and Wnt signalling being the three attracting the most interest. The finding that DISC1 interacts with and inhibits the activity of GSK3β (Mao et al., 2009), and the potential of a dynamic regulatory complex being formed with PDE4B, is highly exciting, and may begin to explain some of the phenotypic findings seen in DISC1 mutant mice.

1.8 DISC1 and signal transduction

siRNA knockdown of DISC1 in primary cortical cultures results in significantly decreased phosphorylation of ERK1/2 (pERK) and AKT (pAKT) (Hashimoto et al., 2006). Viral infection of both the Ser704 and Cys variants of DISC1 results in an increase in pERK1/2 and pAKT. These effects are more significant when the Ser variant is overexpressed: the pAKT increase after overexpression of the Cys variant is non-significant (Hashimoto et al., 2006). In rescue experiments, overexpression of Ser DISC1 variant compensated for the decrease in pERK1/2 resulting from siRNA mediated DISC1 knockdown. There was a non-significant return to basal pERK1/2 levels when the Cys variant was overexpressed: a similar pattern was observed with pAKT (Hashimoto et al., 2006). Thus, it seems that DISC1 plays a similar biological role to that of mood stabilisers in this paradigm, and is capable of enhancing ERK and AKT activation, with the potential to exert effects on synaptic plasticity and neuron survival as described earlier.

Affinity chromatography, immunoprecipitation and *in vitro* binding assays demonstrate that DISC1 forms a ternary complex with Kinesin-1 and Grb2, an adaptor molecule linking neurotrophin binding at tyrosine kinase receptors to downstream effectors such as Ras by recruiting guanine-nucleotide exchange factors (Shinoda et al., 2007). Ras

acts upstream of AKT and ERK in the MAPK and PI3K signalling pathways (Reichardt, 2006). DISC1 and Grb2 are partially colocalised along microtubules in the central region of primary rat hippocampal axon growth cones: DISC1 knock down prevents Grb2 from accumulating in this area. Growth cone localisation of Grb2 is rescued by overexpression of wild type DISC1, but not by overexpression of DISC1 with a mutated SH3 binding domain (Shinoda et al., 2007). However, unlike the observations described above in unstimulated neurons, knockdown of DISC1 has no effect on ERK2 phosphorylation in neurotrophin-3 (NT-3) treated PC12 cells. Knockdown of Grb2 results in decreased pERK2. The story in neurons is more complex: DISC1 knockdown causes decreased levels of pERK1/2 in response to NT-3 treatment, but only in the growth cone, not the cell body. Under NT-3 stimulated conditions, DISC1 knock down results in an axon elongation defect which is not rescued by overexpression of the SH3 domain mutated DISC1 (Shinoda et al., 2007). Conversely, the axon elongation defect seen in non-NT-3 stimulated neurons is rescued by both wild type and mutated DISC1, suggesting that the DISC1/Grb2 interaction, and thus localisation of Grb2 to the growth cone, is only critical for NT-3 stimulated axon elongation (Shinoda et al., 2007). This works suggests a possible pathway by which DISC1 may alter ERK and AKT phosphorylation, but there are some discrepancies between the two pieces of work which have yet to be resolved. It may be that the differences between the effects of DISC1 observed in unstimulated neurons arise from methodological differences (such as differences in cortical versus hippocampal neurons), or the situation may be more complex than is presently appreciated.

Knockdown of DISC1 in the adult mouse brain results in a cellular phenotype very similar to that of PTEN suppressed mice (Kwon et al., 2006). Adult born neurons in the dentate gyrus microinjected with a DISC1 shRNA containing oncoretrovirus have larger cell bodies, an increased number of ectopic primary dendrites, more complex dendritic arborisation and an accelerated rate of synapse formation (Duan et al., 2007). Whereas adult born neurons normally integrate solely within the inner two thirds of the dentate gyrus granule cell layer, most DISC1 knockdown neurons had migrated to the outer

third of the granule cell layer by two weeks post injection, with some penetrating as far as the molecular layer. At 4 weeks post injection, 50% of DISC1 knockdown neurons had reached the molecular layer whereas strikingly, no control cells were present in this layer (Duan et al., 2007). In the PTEN mutant mice, knockdown was limited to post mitotic neurons, due to the NSE promoter used, but despite this a very similar cellular phenotype to DISC1 knockdown is observed. PTEN negative dentate gyrus neurons have increased soma size, an increased number of ectopic processes which project across a larger area, and a highly significant increase in synapsinI staining and the number of presynaptic vesicles. Importantly, cells with ectopic dendrites exhibited an increase in pAKT and phosphorylated GSK3β, whereas cells without ectopic dendrites had no pAKT changes (Kwon et al., 2006).

This phenotypic similarity led to the hypothesis that DISC1 regulates the developmental of adult born neurons through activation of AKT signalling. DISC1 directly binds KIAA1212, a protein which is expressed during differentiation of adult born neurons, and binds to AKT to potentiate AKT activation. The mechanism by which KIAA1212 achieves this remains unclear, but the protein exhibits no kinase activity (Anai et al., 2005). DISC1 single cell knockdown results in an increased immunostaining of pAKT in immature post mitotic neurons. Concomitant elevation of pS6, a downstream marker of activated AKT signalling is also observed. Treatment of HEK293 cells with insulin activates AKT signalling. Overexpression of KIAA1212 in this system is sufficient to elevate levels of pAKT and pS6 without insulin stimulation. Coexpression of DISC1 and KIAA1212 decreases KIAA1212 phosphorylation resulting in decreased pAKT and pS6 in both experimental situations. Overexpression of DISC1 alone had no effect on pAKT levels in either stimulated or unstimulated cells (Kim et al., 2009b). This is in contrast to earlier observations in cortical neurons, where DISC1 overexpression in unstimulated cells resulted in an increase in pAKT (Hashimoto et al., 2006). Immunoprecipitation experiments suggest that coexpression of DISC1 and KIAA1212 decreases the association of KIAA1212 with endogenous AKT, an association which is constant in the absence of excess DISC1, regardless of cell stimulation. There is no

direct binding between DISC1 and AKT (Kim et al., 2009b). KIAA1212 overexpressing neurons, (thus increasing AKT signalling) exhibit a cellular morphology and dendritic arborisation strikingly similar to PTEN and DISC1 knockdown neurons, as do neurons expressing a constitutively active form of AKT. Both these neuron types exhibited a migration and integration phenotype, but less severe than is observed with DISC1 knockdown (Kim et al., 2009b). Rapamycin can be used to inhibit mTOR, a kinase enzyme that functions downstream of AKT. mTOR regulates translation by phosphorylating eIF-4E. Rapamycin application fully rescued the effects of DISC1 knockdown or KIAA1212 overexpression in adult born neurons, whilst having no effect on S6 phosphorylation or morphology in control cells. Application of SB216763, a specific inhibitor of GSK3β, had no significant rescue effect, implicating mTOR as the pathway of interest with respect to differentiation, growth and integration of adult born neurons (Kim et al., 2009b).

1.8.1 DISC1 and adult neurogenesis: a brief summary

Adult hippocampal neurogenesis arises from neural progenitor cells in the subgranular zone that give rise to functional granular neurons that fully integrate into the dentate gyrus. This process is of great interest to the study of psychiatric disease, as various rodent models of major depression, stress and schizophrenia have all resulted in decreased proliferation of neural progenitors, and decreased survival of mature adult born neurons (DeCarolis and Eisch). Treatment with a large range of psychoactive drugs (antidepressants, NMDA antagonists and neuroleptics: though there is more controversy over the precise effects of antipsychotics) and exercise generally has the opposite effect, resulting in an increased pool of amplifying progenitor cells, and an increased number of post mitotic neuroblasts and mature neurons. In humans, major depression, post traumatic stress disorder, Alzheimer's Disease and schizophrenia have all been linked to decreased hippocampal volume and aberrant function. This decrease in volume however does not appear to relate directly to a loss of adult born neurons, and should be approached with some caution, particularly as it is unclear whether possessing

a smaller hippocampus is actually a risk factor for these diseases (for an introductory review of neurogenesis see DeCarolis and Eisch, Duman, 2002). Studies of DISC1 add weight to the interest in this field. As described above, DISC1 has a dual pronged effect on the various stages of neurogenesis. DISC1 acts via regulation of GSK3β and Wnt signalling to have effects on proliferation of neural progenitor cells, while it functions upstream of AKT and mTOR signalling to regulate growth, morphology and integration of adult born neurons. Once more, DISC1 appears to be involved in the integration of extracellular signals and fine control of signal transduction which is vital for normal neuronal location, connectivity and brain function.

1.9 Phosphodiesterase-4

The PDEs are a large family of enzymes which form the sole means of inactivating cAMP within the cell (Alberini et al., 1995, Duman, 2002), playing numerous roles in compartmentalised cellular signalling and signal transduction (Lynch et al., 2006, Houslay, 2010). cAMP is the diffusible downstream signalling molecule of a wide variety of G-protein coupled receptors (GPCRs), and well controlled fluxes in cAMP distribution are vital for maintaining appropriate intracellular signalling (Lynch et al., 2006). A complex pattern of splicing leads to their being more than 20 PDE4 isoforms, all possessing unique N-termini (Houslay and Adams, 2003). Some PDE4 subtypes, including PDE4B2 and one or both of PDE4D1/D2 have gene promoters which are responsive to local cAMP concentrations (D'Sa et al., 2002). All PDE4 long forms have the same basic structure. They possess a unique N-terminus involved in localisation of the enzyme, a regulatory module consisting of an upstream conserved regions 1 (UCR1) and UCR2 domain, whose organisation can be disrupted by PKA phosphorylation, and a catalytic domain. All short forms lack UCR1. PKA phosphorylation in the UCR1 domain of long forms opens the PDE4 structure, increasing enzyme activity, while ERK is capable of phosphorylating UCR2 in PDE4B, C and D. PDE4A lacks the ERK

consensus site. Whether this phosphorylation is inhibitory or activating depends upon the PDE4 subtype (Baillie et al., 2000, MacKenzie et al., 2002, Burgin et al., 2010).

PDE4s are highly expressed in the human brain. PDE4A and PDE4D are moderately expressed in the frontal cortex. PDE4B and PDE4C are detectable here, but at low levels. Binding assays show a high density of rolipram binding sites throughout the cortex (Pérez-Torres et al., 2000). In the hippocampus, PDE4A, PDE4B and PDE4D are expressed highly throughout the dentate gyrus and CA2, with PDE4A being decreased in CA1 and CA3. Again, high rolipram binding is exhibited throughout. Expression in the rodent is similar, with the major difference being that PDE4C expression is restricted to the olfactory bulb (Pérez-Torres et al., 2000). As with DISC1, there are multiple levels of evidence implicating PDE4s in psychiatric disease, from genetic association with human disease to pharmacological and genetic models in rodents.

1.9.1 Genetic evidence linking PDE4 to psychiatric disease

Systematic screening of schizophrenia and bipolar disorder probands for cytogenetic rearrangements highlights PDE4B as an independent risk factor for schizophrenia. A schizophrenic Scottish proband and related cousin with a psychotic disorder carry a balanced translocation at t(1;16)(p31.2;q21), interrupting the gene coding for PDE4B, and predicted to affect production of the PDE4B1 isoform (Millar et al., 2005b). Association studies to be discussed below show some positive findings for the PDE4 genes and schizophrenia, but as with DISC1, there are also some negative studies. However, two main regions of interest for psychiatric disease seem to be appearing in PDE4B: the central region of intron 3, and intron 7, which lies close to the PDE4B2 splice site (Pickard et al., 2007, Fatemi et al., 2008).

Positive association between a series of SNPs in PDE4B and schizophrenia is reported in a Caucasian population (Fatemi et al., 2008). A haplotype based on two of these SNPs is also associated with schizophrenia in this population. These SNPs are clustered in

intron 7 near the splice site for the PDE4B2 short isoform. Positive associations within this region but with different SNPs are also seen in an African American population (Fatemi et al., 2008). This region also holds significant association with schizophrenia in a Japanese population (Numata et al., 2008). Decreases in PDE4B isoforms are found in post mortem cerebellar tissue from the Stanley foundation, but this decrease is only significant for the PDE4B4 and PDE4B2 isoforms (Fatemi et al., 2008). A trend towards a similar decrease is reported from frontal cortex samples. Tissue from bipolar patients also showed a significant decrease in cerebellar PDE4B3 (Fatemi et al., 2008).

The presence of a protective 7 SNP haplotype found within intron 3 of PDE4B is also reported, which reaches significance in females only (Pickard et al., 2007). A Finnish study also finds this region to be of interest, reporting a single risk SNP and the neighbouring haplotype block as having protective and risk forms (Tomppo et al., 2009). A Scandinavian study finds a cluster of SNPs near the PDE4B3 splice site that are nominally associated with schizophrenia and bipolar disorder, but fail to survive multiple testing (Kähler et al.) However, post hoc analysis showed 4 of these SNPs do reach significance, but in females only, and 4 are related to positive symptoms of schizophrenia (Kähler et al.). A further small study finds no evidence of association of PDE4B with schizophrenia, and no evidence of an interaction between PDE4B and DISC1 SNPs (Rastogi et al., 2009).

It seems that fewer genetic studies have been performed on PDE4A and PDE4D, and I was only able to find one positive report. This finds association with a single SNP and an associated haplotype in the final intron of PDE4D, which is over represented in schizophrenic individuals (Tomppo et al., 2009). The is also weak GWAS evidence for association of a PDE4D SNP with a form of neuroticism (Shifman et al., 2007). It is clear that further study is required to adequately assess the genetic risk of PDE4A and PDE4D to schizophrenia, but early results for PDE4B are promising.

1.9.2 Pharmacological evidence for an involvement of the PDE4s with psychiatric disease

Carefully regulated distribution of cAMP is important in many processes in the brain, including memory and cognition, both processes which are altered in schizophrenics with respect to the general population. First generation antipsychotics increase cAMP *in vitro* and *in vivo*, whereas second generation antipsychotics have differential effects, probably reflecting the profile of receptors they target (Molteni et al., 2009). Chronic haloperidol treatment increases PKA activity in the striatum, but has no effect in the hippocampus or cortex. Chronic clozapine decreases PKA activity in all three areas, and alters the expression of PKA subunits (Dwivedi et al., 2002).

Few experiments have been performed to assess putative anti-psychotic or mood altering effects of PDE4 inhibition. In wild type mice, treatment with chronic rolipram, a PDE4 inhibitor, has anxiolytic and antidepressant effects. Rolipram treated mice increase the time in, and number of entries to, open arms of the elevated plus maze, show decreased latency to enter a light compartment, and once there spend more time in the light. These effects are markedly similar to those of the tranquilising benzodiazapine diazepam. Treated rodents also show significantly less immobile time in the forced swim and tail suspension tests (Li et al., 2009). Rolipram increases cAMP concentration and phosphorylation of CREB in the hippocampus and PFC, and increases the rate of neurogenesis in the hippocampus. The anxiolytic effects of chronic rolipram treatment are ablated by treatment with MAM, a DNA methylating agent that blocks neurogenesis (Li et al., 2009), suggesting PDE4 produces at least a subsection of its neurological effects by mediating neurogenesis.

In mice, acute rolipram treatment antagonises hyperlocomotion induced by PCP and D-amphetamine, in a similar way to the licensed anti-psychotics haloperidol and risperidone (Siuciak et al., 2007). Chronic treatment of mice with antidepressants (the noradrenaline reuptake inhibitor Desipramine, serotonin reuptake inhibitors fluoxetine and sertraline, a monooxidase inhibitor and chronic electroconvulsive stimuli) leads to

changes in PDE4 subtype expression. PDE4A and PDE4B mRNA and protein expression was increased in the frontal and parietal cortex, but not in the hippocampi. PDE4B mRNA and protein is increased in response to antidepressant treatment, and decreased by cocaine in the nucleus accumbens, an area associated with reward behaviour (Takahashi et al., 2009). Chronic clozapine treatment in mice increases PDE4B2 and PDE4B4 mRNA and protein expression in striatal membrane preparations (Dlaboga et al., 2008).

The majority of experiments performed using pharmacological manipulation of PDE4 have been centred on cognitive function and memory, as opposed to antidepressant effects. Reneerkens has written a highly detailed review on all such experiments performed thus far, so I will only summarise the recurring themes here (Reneerkens et al., 2009). Acute treatment of wild type rodents with rolipram leads to improvements in memory consolidation and spatial working memory, even in aged mice. Monkeys improve in executive function paradigms after a single dose 30 minutes before testing. (Reneerkens et al., 2009). In aged monkeys however, rolipram actually has a negative effect on working memory, an opposite effect to that seen in young monkeys (Ramos et al., 2003). It is possible to induce memory deficits in rodent models through a wide range of protocols. MEK/ERK inhibition, treatment with the muscarinic antagonist scopolamine and non competitive NMDA antagonist MK801 leads to two forms of deficit in spatial working memory as measured by the radial arm maze (Zhang and O'Donnell, 2000, Zhang et al., 2000, Zhang et al., 2004). These treatments increase the number of working memory errors, so that mice re-enter arms of the maze they have already visited, and reference errors, where mice enter unbaited arms(Zhang et al., 2000). Forskolin treatment shows a trend towards reversal of the scopolamine induced deficit, but this effect was non-significant (Zhang and O'Donnell, 2000). The drug induced increase in both these error types is attenuated by acute rolipram treatment (Zhang and O'Donnell, 2000). This implies that the increase in cAMP resulting from PDE4 inhibition relates to specific areas of the brain. A widescale increase resulting from forskolin treatment may be beneficial in some brain areas but disruptive in others.

Treatment of mice with MK801 also leads to deficits in long term memory leading to decreased latency in an inhibitory avoidance task which is rescued by rolipram administration (Zhang et al., 2000).

Recent work by Vecsey *et al* focusses on sleep deprivation in mice, and demonstrates that two cAMP dependant LTP paradigms (spaced 4 train or theta burst) are impaired after 5 hours of sleep deprivation in mice. Long term maintenance of LTP induced by forskolin treatment is also impaired in these mice, and brain slices show decreased levels of cAMP in CA1, both at baseline and after forskolin treatment, and decreased phospho-CREB. These deficits are rescued by treatment with IBMX, a non-specific PDE inhibitor, suggesting that PDE activity may be increased after sleep deprivation. Indeed, PDE4 possesses increased activity after sleep deprivation, and the PDE4A5 isoform is upregulated in these mice. Thus rolipram treatment successfully rescues the LTP deficit induced in these mice, and also rescues sleep deprived deficits in context specific memory in a fear conditioning paradigm (Vecsey et al., 2009).

The beneficial effect of PDE4 inhibition extends to other conditions that also have a significant cognitive component, especially ones involving memory. Mouse models of Rubenstein-Taybi syndrome, a rare disorder which results from heterozygous mutation of the CREB binding protein (CBP), have intact learning and short term memory, but defective long term memory as characterised by fear condition and object recognition paradigms. A single infusion of rolipram into the hippocampi returns the 1 day memory of these CBP mutant mice to maximal levels (Bourtchouladze et al., 2003).

One of the more common models of Alzheimer's disease, a disease characterised by severe progressive memory loss involves expression of mutant amyloid precursor and presenilin-1 protein. This model recapitulates aspects of Alzheimer's memory loss, with impaired LTP and synapse function, and deficits in spatial working memory (Gong et al., 2004). Acute rolipram treatment almost entirely rescues the LTP deficit in hippocampal slices from these mutant mice, and deficits in freezing behaviour but has

no effect on spatial working memory (Gong et al., 2004). When rolipram was administered daily for 3 weeks, then withdrawn for 6-8 weeks, the benefits of rolipram were maintained, and improvement was also seen in the radial arm maze, a measure of spatial working memory. These deficits may be due to a decrease in active CREB, thought to be required for LTP, as phospho-CREB is lower in the hippocampi of mutant mice. This effect is also rescued by rolipram treatment (Gong et al., 2004). Thus, inhibition of PDE4 is effective in treating memory disorders that are likely to have distinct etiological routes to schizophrenia, suggesting the potential for these routes to lead to a common pathway.

1.9.3 Evidence from knockout rodent models – the contribution of specific isoforms to PDE4 function

Due to the lack of PDE4 subtype specific inhibitors being available, some groups have produced knockdown mice to assess the contribution of PDE4 subtypes to the effects observed with rolipram. Mice deficient in PDE4B display no gross abnormalities, but their weight diverges from that of wild type mice after 21 weeks of age. In behavioural testing, knockout mice show decreased immobile time in the forced swim test (Siuciak et al., 2008, Zhang et al., 2008). No change in memory related behaviour and the passive avoidance paradigm (suppression of preference to avoid shock) is apparent (Siuciak et al., 2008, Zhang et al., 2008). However, PDE4B knockout mice show increased anxiety in an array of paradigms, including decreased head dips at the hole board, an increased latency to cross to a light compartment, decreased exploration in an open field and have increased serum levels of corticosterone (Zhang et al., 2008). Other phenotypes including an increased amplitude of startle response, PPI deficits, increased sensitivity to amphetamine stimulated hyperlocomotion are also reported (Siuciak et al., 2007). Biochemical measurements suggest PDE4B knockouts have a hypodopaminergic striatum (decreased dopamine, and decreased metabolites DOPAC and HVA) with decreased serotonin metabolites and turnover. In the hippocampus however, serotonergic tone is increased with a decreased turnover (Siuciak et al., 2008). These

experiments suggest that knocking out PDE4B has a mild antidepressant effect, a strong anxiogenic effect and significant effects on the information filtering.

Conditioned Avoidance Responding (CAR) is a behavioural test commonly used to predict the efficacy of a putative antipsychotic. In this somewhat counter intuitive paradigm, drugs possessing antipsychotic activity inhibit a trained rodent's avoidance of a shock in response to a stimuli, but not the actual response. Drugs without antipsychotic efficacy either have no effect, or suppress both the avoidance behaviour and the response to shock (Wadenberg and Hicks, 1999). Treatment of wild type rats with rolipram dose-dependently inhibits CAR in rats. However, PDE4B knockout mice are less sensitive to rolipram treatment in this paradigm than wild type mice (Siuciak et al., 2007), suggesting that PDE4B activity is required for mediating the putative antipsychotic effects of rolipram.

PDE4D knockout mice have decreased total PDE4 activity in the cortex and hippocampus (Zhang et al., 2002), agreeing with the finding that PDE4A, PDE4B and PDE4D are all highly expressed in CA1 of the rat, while PDE4A and PDE4D alone are more moderately expressed in CA3 (Pérez-Torres et al., 2000). This experiment suggests that the other PDE4s are not able to completely compensate for the loss of PDE4D in these mice. Indeed, cAMP was found to be increased in cerebral cortical slices from knockout mice (Zhang et al., 2002). PDE4D knockout mice behave as wild-type mice treated with antidepressants, showing decreased immobile time in the tail suspension and forced swim tests. Chronic treatment with rolipram also decreased immobile time in wild type mice, but not PDE4D knockouts. Two antidepressants in clinical use, desipramine and fluoxetine however, still possessed full antidepressant activity in the knockout mice (Zhang et al., 2002). This suggests that rolipram and the two clinically used anti depressants exert their effects through different mechanisms. PDE4D is required only for rolipram to exert its effects. PDE4D knockout mice showed no difference to wild type mice in two anxiety related paradigms: the elevated plus maze and multi-compartment chamber (Zhang et al., 2002).

In a separate experiment, PDE4D knockout mice show decreased freezing time when subject to fear conditioning paradigms. Context and cue dependant fear conditioning is affected, suggesting impaired associative learning. These deficits are only evident 24 hours post training, not in the hour directly post training (Rutten et al., 2008). Recordings from single neurons of PDE4D knockout mice suggest that the loss of PDE4D has no effect on basal synaptic transmission or pre synaptic glutamate release. However, when subject to 2 different LTP inducing protocols, LTP recordings are enhanced, suggesting inappropriate chemical strengthening of synapses when PDE4D is absent. No difference is obtained when LTD protocols are employed (Rutten et al., 2008).

When these genetic, pharmacological and behavioural experiments are taken as a whole, there seems to be no doubt that normal PDE4 function, particularly that of PDE4B and PDE4D, is of great importance to a series of processes key to psychiatric pathology. It is also clear that the full picture is likely to be very complex, a tale of multiple isoforms, varying intracellular and regional localisation, and associating proteins. Elucidating the precise isoforms involved with every aspect of PDE4 function will be a difficult task, but one that should reap huge rewards with regard to psychiatric illness. Isoform specific inhibitors or even genetic knockout models are required. The interaction between DISC1 and the PDE4 enzymes links two highly interesting risk proteins, and may provide insight into how alterations in such widespread proteins lead to such localised deficiencies in function.

1.10 Strategies for treating psychiatric disease

1.10.1 First and Second Generation Antipsychotics: The Dopamine Hypothesis of Schizophrenia

The uniting aspect of all fully licensed antipsychotic drugs is that they possess activity at D2 type dopamine receptors. Originally, neuroleptic activity of first generation antipsychotics was shown to correlate with the capacity to antagonise this receptor (Seeman et al., 1976). Modern PET based binding studies of first episode schizophrenics treated with haloperidol suggest a wide range of DRD2 occupancy by the drug, from 38 to 87% occupancy of available receptors (Kapur et al., 2000). Occupancy of DRD2 predicted the outcome of treatment, but also the likelihood of extra-pyramidal symptoms (parkinsonian symptoms and prolactin elevation). Occupancy above 65% predicted a clinical response, whilst occupancy above 72% and 78% lead to hyperprolactinaemia and EPS respectively (Kapur et al., 2000). Thus there is a rather narrow window for therapeutic efficacy of drugs antagonising DRD2 in schizophrenia. Indeed, many of the second generation antipsychotic drugs possess lower affinity for DRD2 receptors than first generation drugs (Talbot and Laruelle, 2002, Abi-Dargham, 2005): in a study using fixed doses of risperidone and olanzapine, lower dose risperidone was found to be more potent than olanzapine, with DRD2 occupancies of 69 and 55% respectively (Frankle et al., 2004). Second generation antipsychotics tend to possess activity at a wide variety of other neural receptors, particularly 5-HT2A, which may be responsible for their varying efficacy and side effect profiles (Abi-Dargham and Laruelle, 2005).

These findings form the basis of the dopamine hypothesis of schizophrenia, which remained the predominant theory of schizophrenia pathogenesis for many years. Other strands of evidence also add weight to the idea that dopamine dysregulation may lead to schizophrenia, particularly the positive symptoms. Early clinical studies found that the dopamine enhancing agents amphetamine, methylphenidate and L-dopa can all result in acute psychosis (for an introductory review see Abi-Dargham, 2005). Repeated exposure to amphetamine can lead to paranoid psychosis, hallucinations, and progress to

thought disorder (Abi-Dargham, 2005). All these are common features of a psychotic episode in schizophrenia. Treatment with these substances leads to sensitisation to future dopamine release, leading to theories that schizophrenic patients may be endogenously dopamine super sensitive, perhaps through expression of high affinity DRD2 receptors (Philip Seeman, 2006).

While dopamine hyperfunction in the striatum appears to correlate well with the positive symptoms of schizophrenia, these models fail to recapitulate the negative symptomology of the disease. The PFC influences striatal dopamine through two functional connections: an activating glutamatergic pathway and an inhibitory GABAergic pathway (Weinberger and Laruelle, 2001). The full dopamine hypothesis is therefore two fold: that hyperactive subcortical mesolimbic projections lead to hyperstimulation of DRD2 in the striatum, resulting in positive symptoms, while dopamine hypoactivity in the PFC leads to hypostimulation of DRD1 in the PFC, causing negative and cognitive symptoms (Abi-Dargham, 2005). In studies of DRD1 availability in the DLPFC, untreated schizophrenics showed increased DRD1 availability which correlated with poor performance in the N-back verbal working memory paradigm (Abi-Dargham et al., 2002).

Postmortem studies in this field have been wide ranging and controversial. With these studies, adaptive changes long term use of neuroleptics must always be carefully considered as a confounding factor. A significant number of studies have found increased striatal DRD2, while some hint at increased subcortical dopamine and metabolites in schizophrenia. Studies generally report no changes in DRD1 or dopamine transporter expression (Abi-Dargham, 2005). This area is clearly one that would benefit from a systematic meta-analysis. Imaging studies in live patients have attempted to further address these questions. In their large meta-analysis of all imaging and binding studies up to 2001, Weinberger and Laruelle found a series of replicated dopaminergic system alterations, the most striking being a small but significant 13% increase in DRD2 receptors in the striatum in schizophrenic patients. Patients also generally showed more

variability in DRD2 expression than healthy controls (Weinberger and Laruelle, 2001). Their literature review suggested increased accumulation of DOPA decarboxylase in the striatum of schizophrenic patients, and an enhanced amphetamine induced decrease in tracer binding. This latter effect was exacerbated during periods of psychosis (Weinberger and Laruelle, 2001). Schizophrenic patients have a higher DRD2 occupancy, as assessed by depletion studies, and occupancy is predictive of a good therapeutic response to 6 weeks of antipsychotic treatment (Abi-Dargham et al., 2000). No consistent changes in DRD1 expression in schizophrenia have been reported, but there is a trend towards low PFC DRD1 expression being associated with negative symptoms and poor Wisconsin Card Sort Test performance (Weinberger and Laruelle, 2001).

Genetic studies have also shown some indication of dopaminergic dysfunction in schizophrenia. The most highly studied of these is a non synonymous Val/Met SNP in catechol-O-methyl transferase, an enzyme involved in dopamine degradation, particularly in the PFC where there is low expression of the dopamine transporter protein (Goldberg and Weinberger, 2004, Williams et al., 2007). Most work has focussed on this Val/Met SNP, which alters stability, and hence activity of the enzyme. Although a recent meta-analysis failed to show a significant association of either variant with schizophrenia (Williams et al., 2007), significant association is found between brain volume changes common in schizophrenia and cognitive ability (van Haren et al., 2008). In just one example, healthy subjects carrying two copies of the more stable higher activity Val allele perform less well in the Wisconsin Card Sort Test and the verbal N-back test (measures of working memory and executive function) than candidates possessing at least one Met allele (Goldberg and Weinberger, 2004). fMRI shows a more “efficient” BOLD response to these tasks in Met allele carriers in normal conditions, but this deteriorated at high working memory loads when they were treated with amphetamine, suggesting that their higher basal dopamine was increased to disruptive levels by amphetamine. Conversely, amphetamine enhanced efficiency in Val/Val individuals, suggesting that they have lower basal PFC dopamine, which is

enhanced to optimal levels by amphetamine treatment (Goldberg and Weinberger, 2004).

While evidence is consistently strong for an involvement of dopamine hyperactivity with the positive aspects of schizophrenia, the evidence for dopamine hypofunction in the PFC is mostly indirect and not yet totally convincing. It is clear however, that dopaminergic tone in the PFC is intricately involved with cognitive function in healthy subjects, and thus may still be important for this complex disease. In addition, first and second generation antispsychotics have an adverse side effect profile including weight gain, hyperporlactinaemia and extra pyramidal side effects, including Parkinsonian like tremors. (Lieberman et al., 2005). These side effects often lead to discontinuation of treatment. In the CATIE trial designed to assess efficacy of first versus second generation antipsychotics, the most striking result is that 74% of the patients in this large, double blinded, multi centre study discontinued medication before the 18 month duration of the project was complete. Olanzapine had the lowest discontinuation rate (64%), but was still associated with significant weight gain and altered metabolic measures. The first generation antipsychotics, as expected from receptor affinity studies, had a greater rate of extra pyramidal symptoms (Lieberman et al., 2005). From this side effect profile alone, it is clear that current treatment for schizophrenia is far from optimal. To add to this, 40-50% of all schizophrenic patients are "treatment resistant," being inadequately responsive to two or more antipsychotic drugs (Daskalakis and George, 2009). In attempts to discover other targets for medication, and produce a pharmacological model of schizophrenia that more fully recapitulates all aspects of the disease, attention turned to glutamate, and a new theory was postulated.

1.10.2 Metabotropic Glutamate agonists: The Glutamate Hypothesis

The glutamate hypothesis of schizophrenia predates the first successful Phase II clinical trials with LY404039, a highly selective agonist of mGlu2/3 receptors in 2005 (Patil et al., 2007). The excitatory functional connections between most brain regions implicated in schizophrenia: the PFC, hippocampus, limbic system, basal ganglia, thalamus and

striatum, are glutamatergic (Moghaddam, 2003). NMDA receptors have been linked to important roles in axon guidance, synaptic pruning, and LTP: all factors which are have also shown abnormalities in models of schizophrenia (Meador-Woodruff and Healy, 2000).

Original evidence for this hypothesis derived from the study of the effects of non competitive NMDA antagonising drugs on human and animal behaviour. In their 1991 review, Javitt and Zukin describe many of these early experiments. In healthy humans, acute PCP treatment induces a state lasting several hours that recapitulates many of the positive and negative features of schizophrenia. Subjects become withdrawn, experience poverty of speech, and thought disorder, and have disrupted cognitive processes. Measures of attention and information processing, such as paired learning and association tests show fronto-temporal and hippocampal functioning to be affected to a similar extent as is observed with schizophrenic patients (Javitt and Zukin, 1991). A later study attempted to more rigorously define the cognitive effects of NMDA antagonism in humans. This showed no deficits in a virtual Morris Water Maze paradigm, suggesting intact acquisition and retrieval of spatial memory. In verbal tasks, ketamine treated individuals show impaired encoding of new words, and poor attention (Rowland et al., 2005). At high doses, PCP administration results in a dissociation from the local environment, but not a loss of consciousness (Javitt and Zukin, 1991). In stable schizophrenic subjects, PCP administration precipitates a psychosis encompassing many of the features of earlier "endogenous" episodes that may last for weeks (Javitt and Zukin, 1991). Ketamine, another non competitive NMDA antagonist has the same effect in schizophrenic subjects (Lahti et al., 1995).

Repeated administration of ketamine over 3 days to healthy human subjects results in a dose related increase in positive and negative symptoms, including altered self perception, hallucinations, paranoia and thought disorder, coupled with withdrawal, detachment and poor WCST performance (Krystal et al., 1994). Velvet monkeys treated with PCP for 14 days develop deficits in object retrieval paradigms, suggesting

compromised executive function (Jentsch et al., 1997). These monkeys had decreased dopamine use in the DLPFC and prelimbic cortex, measured by decreased levels of homovanillic acid. These deficits endured for at least 4 weeks post treatment withdrawal (Jentsch et al., 1997). Mice, rats and cats acutely treated with PCP develop locomotor hyperactivity and stereotypical movements, and have inhibited spatial learning and associative learning (Javitt and Zukin, 1991).

A series of individual trials of allosteric NMDA agonists which act at the glycine modulatory site of the NMDA receptor have shown efficacy in improving both positive and negative symptoms of schizophrenia, when used as an adjunct to established antipsychotic regimes. D-serine shows efficacy as an adjunct in ameliorating positive and negative symptoms, as well as improving performance in the Wisconsin Card Sort Test (Tsai et al., 1998). D-cycloserine showed no cognitive enhancement when used as an adjunct, but did successfully ameliorate negative symptoms in patients with deficit syndrome, a form of schizophrenia with primary, enduring negative symptoms (Goff et al., 1999, Heresco-Levy et al., 1999). Glycine as an adjunct produced positive and negative symptom improvement in treatment resistant schizophrenia (Heresco-Levy et al., 1996, Heresco-Levy et al., 1999). However, a more recent meta-analysis of these trials suggests that significant effects of allosteric NMDA enhancement are limited to a moderate amelioration of negative effects. Glycine and D-serine, are the most effective of these treatments (Tuominen et al., 2005).

Genetic association studies have highlighted a series of genes associated with glutamatergic function and the NMDA receptor in particular as risk genes for schizophrenia. These are reviewed by Harrison (2003) and include D-amino acid oxidase (DAAO), which metabolises D-serine, and G72, which activated DAAO. The association of neuregulin (NRG) with schizophrenia is one of the more established genetic findings, and NRG regulates NMDA receptor expression through the ErbB4 receptor. Regulator of G protein signalling 4 (RGS4) is a negative regulator of a series of GPCRs, including the metabotropic glutamate receptors, while dysbindin is involved

in the maintenance of synapses (Harrison and Owen, 2003). Finally, a meta-analysis of association studies of the GRIN2B gene, which codes for the NR2B subject of the NMDA receptor, have shown a significant but small association of this gene with schizophrenia (Li and He, 2007).

Postmortem studies describe decreased excitatory phosphorylation of the NR1 subunit of the NMDA receptor in the frontal cortex and hippocampus in schizophrenia, but no change in bipolar disorder or major depressive disorder (Emamian et al., 2004b). Phosphorylation at this residue was increased by treatment with haloperidol in rats *in vivo* and in striatal primary culture (Leveque et al., 2000). There have been many studies of total NMDA receptor expression, but no consistent results in any brain region (Meador-Woodruff and Healy, 2000). I was able to find no evidence of a meta-analysis of these results, as was performed for DRD2 receptor expression (Weinberger and Laruelle, 2001). There does however, appear to be a consistent decrease in protein and mRNA expression of the gluR1 and gluR2 subunits of the AMPA receptor in schizophrenic hippocampi, with decreased tracer binding to this receptor (Meador-Woodruff and Healy, 2000). Tracers for *in vivo* binding assays of NMDA receptors have only recently been developed, but early studies show decreased NMDA receptor binding availability in the left hippocampus of medication free schizophrenics. This deficit seems to be compensated for by antipsychotic treatment (Pilowsky et al., 2005).

When all this evidence is brought together, a hypothesis of glutamatergic hypofunction in the PFC resulting in schizophrenia seems very sensible. However, until recently, this rather convincing set of evidence had failed to come up with any great therapeutic benefit. Allosteric modulators acting at the glycine modulatory site show a mild improvement in negative symptoms when used as an adjunct, but are still not in common use (Tuominen et al., 2005). In 2005 however, an mGlu2/3 receptor agonist reached Phase II clinical trials. Electron microscopy suggests varying expression of mGlu2/3 in the brain, including pre-synaptically on glutamatergic corticostriatal neurons (Petralia et al., 1996). Activation of these receptors with mGlu2/3 agonists results in depression of

corticostriatal synapses through a decrease in cAMP (Lovinger and McCool, 1995), giving them an important inhibitory function in transmission in this pathway.

LY404039, a highly specific mGlu2/3 agonist, and olanzapine, a second generation antipsychotic, inhibit PCP induced locomotion in wild type rats. In mGlu2/3 knockout rats, LY404039 had no inhibitory effect, suggesting that mGlu2/3 agonists and olanzapine act through a different pathway to achieve these effects (Patil et al., 2007). In a randomised and double blinded placebo controlled Phase II trial, LY404039 proved to be safe and well tolerated, without inducing the extra pyramidal side effects or weight gain commonly seen with DRD2 antagonising antipsychotics. Reported side effects were generally mild, including insomnia, nausea, headache and drowsiness (Patil et al., 2007). Patients treated with LY404039 showed significant improvements above placebo in positive and negative symptoms, that began after 1 week of treatment, and continued for at least 4 weeks (Patil et al., 2007).

The agonist in question is likely to potentiate, and not activate the mGlu2/3 receptor (Johnson et al., 2005), which may be hypofunctional under conditions of a hypoglutamatergic PFC. This produces a rather counter intuitive picture, where hypofunction of glutamate at the mGlu2/3 receptor in the PFC leads to increased glutamatergic output elsewhere in the brain. Thus compartmentalisation of synapses and location of cortical connections are crucial in determining the functional outcome. Because the drug appears to potentiate activity at this receptor, and not stimulate activity without endogenous activation, a more endogenous approach to therapy is allowed (Johnson et al., 2005). It will be interesting to see how this therapy compares with standard antipsychotic treatment in future trials.

1.10.3 Bringing dopamine and glutamate together

There is currently a chicken and egg style debate over which dysregulation, dopaminergic or glutamatergic, is the causative factor in schizophrenia. While the

intricacies of this argument could fill a thesis alone, it is clear that the two pathways do not act in isolation. Glutamatergic function in the PFC is intricately linked to dopamine transmission in the PFC and striatum. Treatment of the contralateral PFC with tetrodotoxin, a general sodium channel blocking neurotoxin, leads to decreased dopamine release in the medial striatum (Mignon, 1996). Blockade of the tonic GABAergic input to the striatum using bicuculline results in increased dopaminergic tone in the medial striatum (Mignon, 1996), suggesting the presence of both an excitatory and inhibitory input from the PFC to dopaminergic tone in the striatum (Abi-Dargham, 2005). Chronic users of ketamine have increased DRD1 availability in the dorso-lateral PFC (Narendran et al., 2005). In healthy subjects, ketamine administration leads to a greater decrease in tracer binding on amphetamine treatment, implying enhanced dopamine release (Kegeles et al., 2000)

The mGlu2/3 agonist allows for further insight into possible connections between these two theories. As previously discussed, hypoglutamatergic conditions in the PFC may lead to hypofunction of presynaptic mGlu2/3 on corticostriatal afferent connections. This may lead to increased excitation of these connections, and thus aberrant excitation of dopaminergic tone in the striatum. Further work is needed to truly establish the importance and the causation of dysfunction in these pathways in schizophrenia, and establish a hierarchy of effects.

What is clear thus far, is that all these pathways are interconnected, that there is a potential role for numerous neurotransmitters (I have not covered serotoninergic and GABAergic connections in this review, both of whom have a large amount of literature linking them to psychiatric disorder) (Benes, 2002, Aghajanian and Marek, 2000), and that a small malfunction in one component of their downstream circuitry may lead to more wide scale disarray, that manifests as psychiatric disorder. For this reason, a more physiological target for psychiatric disease management may be the molecules that mediate the crosstalk between these pathways, allowing for activity dependant modulating of risk pathways, such as the mGlu2/3 receptor agonist, the PDE4s and

GSK3β. As evidence for schizophrenia risk variants in genes increases, more of these mediating proteins and pathways may be highlighted. This may allow a refinement of our approach to diagnosing psychiatric disease, being able to define subtypes of psychiatric disorder resulting from the actions of a combination of specific risk factors. The ultimate goal of redefining psychiatric disease through these principles lies in the potential for personalised medication, designed to correct specific deficits in a highly localised fashion, thus possessing a less damaging side effect profile and leading to better compliance than the drugs currently in use. Approaches such as this are just beginning to show therapeutic value in other complex diseases such as breast cancer (Huber et al., 2009).

1.10.4 Phosphodiesterase inhibition

Phosphodiesterase inhibitors are already widely used therapeutically in treatment of vascular (Feldman and McNamara, 2002) and inflammatory diseases, as well as cognitive disorders (Rose et al., 2005). The efficacy of highly successful subtype specific inhibitors has been demonstrated by the use of PDE5 inhibitors in the treatment of male erectile dysfunction (Burnett, 2005) and PDE3 inhibitors for the specific treatment of intermittent claudication (Doggrell and Doggrell, 2001). Recent research by Menniti *et al,* 2007, has highlighted a possible role for specific inhibitors of the dual substrate PDE10A in the treatment of psychosis related to schizophrenia, which they attribute to alterations in cAMP mediated activity in medium spiny neurons in the basal ganglia pathways which form a "brake" for behaviour (Menniti et al., 2007). They also cite potential positive effects on the cognitive component of schizophrenia after early mouse studies on PCP induced deficits in extra dimensional "set shifting" (a measure of attentional selection processes), which are attributed to improved corticostriatal neurotransmission on PDE10A inhibitor treatment (Rodefer et al., 2005). The Authors suggest that PDE10A treatment could potentially have an effect on improving executive function in schizophrenic patients.

Rolipram is an antidepressant that specifically inhibits PDE4s via occupation of much of the cAMP binding site (Xu et al., 2004) which reached Phase II of Clinical Trials in 1983 as an anti depressant. Little investigation has been done into its specific use as a treatment in schizophrenia, despite suggestions that people with schizophrenia have altered cAMP mediated signal transduction in platelets and post mortem brain fractions (Robichaud et al. 1999). The lack of investigation is mainly due to the common side effects of Rolipram administration, the most limiting being emesis (Dyke and Montana, 2002) and sedation (O'Donnell and Zhang, 2004). The drug also has a narrow therapeutic window. It is thought that these side effects are mediated at least partly centrally through the action of Rolipram on PDE4D, which is highly expressed in the squirrel monkey (a useful model as rats and mice do not vomit) in the area postrema and neurons of the nucleus tractus solitarius and locus coeruleus, areas involved in triggering emesis (Lamontagne et al., 2001).

There are multiple challenges ahead for the search for clinically useful subtype specific PDE inhibitors. There is still a huge lack of understanding over the subtle differences in expression pattern, localisation and function of not only the PDE4B and PDE4D isoforms, but also the long and short variably spliced subtypes of each isoform. Where these complex patterns fit into the signalling pathways potentially mediating schizophrenia is another step beyond this. With largely similar structures, refining compounds to specifically alter the activity of one isoform is difficult. Finally, PDE4s are known to have two different states, the high and low-affinity rolipram binding states (termed HARBS and LARBS respectively). The HARBS is almost exclusively found in the brain, particularly the hippocampus, frontal cortex and olfactory bulb. The LARBS however is found in peripheral tissues as well as the brain (Zhao et al., 2003). Initial results from recent experiments by Zhang *et al* suggest that the HARBS is most important in mediating the anti depressant effects resulting from Rolipram, with PDE4 inhibitors CDP840, MEM1018 and MEM1091, which have a lower affinity for the HARBS conformation, requiring a five fold dosage to produce effects comparable to

Rolipram in the Forced Swim Test. These inhibitors completely failed to produce an effect in the Differential Reinforcement of Low Rate 72-s operant schedule (Zhang et al., 2006). Both these commonly used animal models are sensitive to antidepressant drugs.

Recently, allosteric modulators that target the UCR2 domain of PDE4 have been developed. In PDE4D, at residue 196, a phenylalanine protrudes from UCR2 and extends towards the active site. This residue is a tyrosine residue in PDE4A, B, and C, and is important in binding of allosteric modulators. This difference in charge can be capitalised on, and selective inhibitors developed with PDE4D selectivity . To this end, an allosteric modulator has been developed with PDE4D selectivity (Burgin et al., 2010) that performs more potently than rolipram in augmenting long term memory and in rescuing a scopolamine induced deficit in spatial working memory. Importantly, this inhibitor is 3000 times less emetic than Rolipram in beagle dogs, and 500 times less emetic in cynomolgus monkeys (Burgin et al., 2010).

1.11 Aims of this PhD

In this introduction I have detailed a number of pathways implicated in the development of not only schizophrenia, but also bipolar disorder and depression, including neurogenesis, neurotrophin, glutamatergic and dopaminergic signalling. Central to all of these pathways is the integration of series of extracellular signals acting at neuronal receptors, to produce an output appropriate for the cellular context and intended purpose. cAMP acts as a key signalling molecule downstream of all of these receptors, and cAMP fluctuations are key for precise control of downstream effects, including gene expression and synaptic transmission. DISC1 has also been linked to all of these conditions, through genetic, animal and in vitro studies, and seems likely to act as part of a common mechanism for susceptibility to psychiatric disease. DISC1 interacts dynamically with the PDE4 enzyme family, two of whom are heavily linked to psychiatric disease by

genetic and pharmacological studies. The PDE enzymes form the only means of inactivating cAMP fluxes within a cell. There are also indications that DISC1 may bind and inhibit GSK3β, a further important signalling enzyme, perhaps as part of a complex involving PDE4. The aim of my PhD therefore, is to investigate the hypothesis that DISC1 and GSK3β may be involved in regulating PDE4 activity, and thus play a role in modulating cAMP signalling downstream from GPCRs. The thesis will therefore address these key points:

It has been established that DISC1 binds PDE4 in a dynamic and isoform specific fashion. I will first describe establishment of a model cellular system suitable for assaying PDE4 activity. I will then discuss examination of the localisation of DISC1 and PDE4B and D, and quantification of the expression of PDE4 isoforms in DISC1 overexpressing and DISC1 knockdown cell lines (chapter 3).

Secondly, I will describe the results of my assessment of the functional effects of the DISC1-PDE4 interaction in these cell lines, under basal and forskolin stimulated conditions Furthermore, I will discuss use of pharmacological inhibition of GSK3β to assess the effect it may have on PDE4 activity (chapter 4). As before, this assay was performed under basal and stimulated conditions, in the presence of excess, or the absence of DISC1 (chapter 5).

Thirdly, I describe investigation of the potential role of PDE4 downstream from two receptors implicated in psychiatric disease: the NMDA and DRD1 receptors. Indirect evidence assessing cAMP accumulation in cortical slices has implicated PDE4 in cAMP inactivation downstream of NMDA receptor agonism. I have assessed the suitability of using primary cortical neurons as a model to measure PDE4 activity. I have characterised PDE expression in these cells using PCR, specific inhibitors of non-PDE4 PDE enzymes, and then assayed PDE4 activity in response to treatment with NMDA and the DRD1 specific agonist A68930. I will also describe investigation of PDE4 activity in primary cortical neurons prepared from mice carrying point mutations in

DISC1. Finally, I have performed immunocytochemistry on these neurons to investigate the localisation of PDE4B, PDE4D and the two receptors of interest (chapter 6).

2 Materials and Methods

2.1 Bioinformatics

2.1.1 Phosphorylation Prediction

Full protein sequences of human PDE4B and PDE4D were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and submitted to a series of phosphorylation prediction databases with varying prediction methods. Information from these databases was combined and tabulated.

2.1.1.1 DISPHOS 1.3

DISPHOS 1.3 (http://core.ist.temple.edu/pred/predhtml) uses information on the disorder of the protein surrounding potential phosphorylation sites to predict potential phosphorylation sites. Predictions are not kinase specific. A score of greater than 0.5 indicates a potential phosphorylation site. DISPHOS 1.3 accuracy is quoted as reaching 81.3% +/- 2.2% for Serine, 74.8% +/- 2.5% for Threonine, and 79.0% +/- 2.4% for Tyrosine (DISPHOS).

2.1.1.2 Scansite 2.0

Scansite 2.0 (http://scansite.mit.edu/) provides kinase specific predictions of phosphorylation potential by comparison with 62 known phosphorylation motifs identified by peptide libraries. Scanning is available at three different stringencies: high, medium and low. The high stringency setting for example, will only report a site that falls within the top 0.2% of scores for that motif within the SwissProt vertebrate database. Medium and low stringency hits will lie within the top 1 and 5% respectively. Graphs showing the surface accessibility of the motif can also be provided (Obenauer et al., 2003).

2.1.1.3 Phosphomotif Finder

Phosphomotif Finder (http://www.hprd.org/PhosphoMotif_finder) searches sequences entered by the user for published kinase specific phosphorylation motifs, with information about the motif sequence and links to the literature for that motif freely available. As such, there is no scoring system (Amanchy et al., 2007).

2.1.1.4 KinasePhos 2.0

KinasePhos 2.0 (http://kinasephos2.mbc.nctu.edu.tw) uses algorithms based on the coupling and distance between amino acids to provide a score relating to the potential for phosphorylation by specific kinases. Predictive accuracy is quoted as 90, 93, 88 and 93% for serine, threonine, tyrosine and histidine respectively (Wong et al., 2007).

2.1.1.5 NetPhosK 1.0

NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/) is trained to identify known kinase specific phosphomotifs, and employs these trained neural networks to identify further sites in sequences supplied by the user. Studies by the developers claim higher predictive sensitivity than Scansite, with an even rate of false positive findings (Nikolaj et al., 2004).

2.2 Cell Culture

2.2.1 Cell Line Maintenance

Cell lines were grown at 37^{o}C, 5% CO_2 in a Galaxy 170S or Galaxy 170 incubator (Scientific Laboratory Supplies). All reagents involved with cell culture were Gibco from Invitrogen. HEK293 and SHSY5Y cells were grown in D-MEM with 10% Foetal Bovine Serum in Cell Star T25, T75 or T175 flasks. Cells were split when they reached 90-95% confluency, on average every 4-7 days. All cell culture work was performed in an Envair Bio2+ Class II Safety Cabinet under containment level 1.

To split cells, old media was aspirated, and cells rinsed twice with warm PBS and once with warm TrypLE. Fresh TrypLE was added and incubated for 2-5 minutes, without removing the flask from the safety cabinet. Cells were detached by tapping the flask, and at least 4 volumes of fresh media were added. Detached cells were then pipetted multiple times to disperse clumps of cells, and removed to a new flask at dilutions of 1:5-1:10 depending on future use.

Where cells were to be used for lysate preparation, a T75 of SHSY5Y cells was generally split 1:5 into T25 flasks. Cells were then grown for 1-3 days to 75-90% confluency, depending on whether transfection or functional assays were required. When cells were to be used for immunocytochemistry, cells were split into individual wells of Costar 12 Well Plates (Corning), each containing a single 16mm sterile glass coverslip (VWR) and 2mls DMEM, and grown until approximately 80% confluency. If cells were to be nucleofected, they were grown to 80% confluency in T75 flasks, then treated as per Nucleofector Kit instructions.

2.2.2 Preparation and Maintenance of Mouse Primary Cortical Neurons

If neurons were being used for immunocytochemistry, then coverslips were pre treated with 100% ethanol, before being heat dry sterilised. One day prior to neuron production, plates were coated overnight with Poly-D-Lysine solution. Plates were washed with distilled water prior to plating neurons. If primary neurons were to be used for PDE assays, then 6 well plates were coated overnight with Poly-D-Lysine solution, and washed with distilled water prior to neuron plating. Washed and dried cover slips and 6 well plates can be stored at 4^{o}C for up to two months.

Pregnant E18 mice (CD1 or C57BL/6) were killed by schedule 1 procedure by trained staff at the animal facility, and then passed to me for dissection. Embryos were dissected out of the mother on ice, and the brains removed to ice cold dissection buffer. Using a Leica MZ6 microscope with a Fiber-Lite MI-150 High Intensity Illuminator, the meninges were peeled from the whole brains, the cortices removed to a petri dish containing new dissection buffer and the hippocampi discarded. Cortices were kept in fresh dissection buffer on ice until all brains were dissected. The dissection buffer was then removed from the cortices, which were added to 0.1% trypsin solution in a Falcon tube (50mls solution per 10 pups). Cortices were incubated in trypsin solution for 45 minutes, before 5 passages through a wide plastic Pasteur pipette. This resulting mixture was centrifuged for 5 minutes at 1500rpm, and the supernatant discarded. The pellet was resuspended in 20mls DMEM with 10% FBS, then passaged 15 times through a wide Pasteur pipette, before a further 5 minutes centrifugation at 1500rpm. Again the supernatant was discarded, the pellet resuspended in 10mls DMEM with 10% FBS, and the solution passaged 20 times through a wide necked, then 10 times through a fine necked plastic Pasteur pipette. A third centrifugation for 5 minutes at 1500rpm was performed, and the pellet resuspended in 10mls DMEM without FBS. The resulting solution was passed through a 40μM cell filter, and the cell number counted using a haemocytometer. After a final centrifugation, the cells were resuspended in Neurobasal medium (plus B27 supplement, Glutamax and Pen/Strep solution) to allow for seeding in

plates at $5x10^4$ cells per well in a 12 well plate, and $6x10^5$ per well in a 6 well plate. Cells were incubated at 37°C, 5% CO_2 in a Galaxy 170 incubator.

For continuing culture, half the growth medium was removed and replaced with fresh medium at 7 days in vitro.

2.2.3 Transfection of plasmids into eukaryotic cell lines

To exogenously express protein in cell line cultures, two methods were used: Lipofectamine 2000 lipofection for HEK 293 cells and stable cell line production in SHSY5Y cells, and Nucleofection for SHSY5Y cells in immunocytochemistry and functional assays. Expression plasmids are detailed where used in the appropriate results chapter.

2.2.3.1 Lipofectamine transfection (Invitrogen)

To transfect SHSY5Y cells for stable cell line production, cells were plated in 10cm cell culture dishes (Iwaki) and grown to approximately 70% confluency. Lipofectamine2000:DNA complexes were made up in room temperature Opti-MEM according to the manufacturer's instructions, containing 24µg of DNA and 60µl Lipofectamine 2000. Old media was aspirated from the plates, and cells were washed twice with warm OptiMEM. Cells were incubated with the lipofection complexes for 6 hours, and the medium replaced by fresh 10% FBS DMEM.

To transfect HEK cells to test DISC1 expression once subcloned into the TO vector, cells were grown to approximately 80% confluency in T25 flasks. Lipofectamine complexes were made up as above, containing 8µg DNA and 20µl Lipofectamine 2000. The procedure was performed as above, and cells were lysed with 200µl REPA buffer the day after transfection for Western Blotting.

2.2.3.2 Nucleofection (Lonza)

Nucleofection was performed according to the manufacturer's protocol, summarised below. 12 well plates or T25 flasks were filled with 1ml DMEM (2 wells of a 12 well plate were used to plate each nucleofection transfection) and pre warmed in an incubator. A T75 flask of SHSY5Y cells was grown to approximately 80% confluency. Cells were detached from the flask by rinsing with warm PBS then TrypLE. Cells were incubated in TrypLE for less than five minutes, and resuspended in fresh DMEM with 10% FBS. Cell concentration was determined using a haemocytometer, and the appropriate number of cells spun in a centrifuge ($1x10^6$ cells per transfection sample for ICC, $2x10^6$ for functional assays, 1000rpm for 5 minutes at 37^oC). Cells were resuspended in 100µl Nucleofector Solution (with supplement added) per transfection reaction. 100µl of cell suspension was combined with 2µg of the required DNA. This mixture was transferred to a sterile Nucleofector cuvette, the cap closed, and placed in the Nucleofector machine. Program A-023 for high cell viability was selected and applied. The cuvette was removed immediately from the machine, and 1ml of warm 10% DMEM added to the mixture. This mixture was then split into 2 wells of a 12 well plate for ICC, or wholly into a T25 flask for functional assays, and could be grown for up to 4 days post transfection.

2.2.3.3 Assessing transfection rate in SHSY5Y cells by FACS scanning

SHSY5Y cells were transfected by both methods described previously with the pmax GFP plasmid supplied in the Nucleofector kit, and grown overnight in 6 well plates. The next morning, cells were detached using TrypLE, then resuspended in PBS for FACS scanning. Cell suspensions were fed into the machine (FACS Calibur, BD Biosciences) using the standard machine protocol, and the number of cells expressing GFP were counted, as a percentage of the total cells counted.

2.2.4 Drug treatment

2.2.4.1 SHSY5Y cells

SHSY5Y cells and stable cell lines based on SHSY5Y (TRTODISC1 DISC1 overexpressing cells and T1 DISC1 knock down cells) were used to examine the effects of common drugs altering the activity of signalling pathways on PDE4 activity. Forskolin increases the intracellular concentration of cAMP through stimulation of adenylyl cyclase, and LiCl/SB216763 inhibits the activity of GSK3β. SHSY5Y cells were grown in T25 cell culture flasks to approximately 90-95% confluency, and then serum starved overnight. The appropriate concentration of drug was then added to the correct flasks the next morning, with a vehicle control of equal volume, as detailed in Table 2.1A After the required treatment time had been completed, cells were rinsed once with ice cold PBS, and lysed with 3T3 lysis buffer for PDE assays to be performed.

Drug	Source	Stock concentration	Final concentration	Vehicle	Treatment time
Forskolin	Sigma	50mM	10μM	1% EtOH	30 mins
Lithium Chloride	Sigma	1M	3/10mM	dH_2O	30 mins
SB216763	Tocris	25mM	10μM	DMSO	30 mins

Table 2.1A: Details of drug treatments performed on SHSY5Y cells.

Drug	Source	Stock concentration	Final concentration	Vehicle	Treatment time
NMDA & Glutamate	Both Sigma	10mM NMDA 100mM Glutamate	10μM 100μM	dH_2O dH_2O	2/5 mins
Lithium Chloride	Sigma	1M	10mM	dH_2O	30 mins
SB216763	Tocris	25mM	10μM	DMSO	30 mins
A68930	Sigma	1nM	1nM	dH_2O	2/5 mins

Table 2.1B: Details of drug treatments performed on primary cortical neurons.

2.2.4.2 Primary neurons

CD1 wild type, Q31L and L100P background control and mutant primary neurons were prepared as earlier described. At 14 days in vitro, pre drug treatment, neurons were starved of B27 supplement and Glutamax (both Invitrogen) for 4 hours in 1ml of neurobasal medium only. When treatment was commenced, a further 1ml of neurobasal

medium containing double the final concentration of drug was added to each well for the appropriate time. Final concentrations and times are detailed in Table 2.1B. Media was then aspirated, neurons rinsed once with ice cold PBS, and lysed with 3T3 buffer for PDE assays to be performed.

2.2.5 Stable cell line production

Previous attempts to produce a constitutively overexpressing DISC1 cell line had proved unsuccessful. While this is interesting in itself, an alternative method of overexpression was needed in the face of low levels of transfection in SHSY5Y cells. I used the Invitrogen T-REx system to produce a stable cell line that would inducibly overexpress DISC1 through the CMV promoter on application of tetracycline.

2.2.5.1 Antibiotic sensitivity testing

In order to select for cells successfully transfected with the pcDNA7/TRex and pcDNA4/TODISC1 plasmids, dual antibiotic selection was required with Zeocin and Blasticidin (both Invitrogen). To this end, antibiotic sensitivity tests were performed on SHSY5Y cells. 6/7 plates of SHSY5Y cells per antibiotic were grown to 25% confluency, and a series of concentrations of Zeocin (0, 50, 125, 250, 500, 750 and 1000μg/ml) and Blasticidin (0, 1, 3, 5, 7.5, 10μg/ml) added to the plates. Selective media was replaced every 3-4 days, and the survival rate of the SHSY5Y cells monitored. Final concentrations of 50μg/ml Zeocin and 2μg/ml were chosen, as these killed approximately 95% of cells after 2 weeks of selection.

2.2.5.2 Stable cell line transfection and selection

SHSY5Y cells were cotransfected with a ratio of 6:1 pcDNA6/TR:pcDNA4TODISC1, using a standard lipofectamine protocol as described in 2.2.3.1. The cells were dual selected in DMEM with 10% foetal bovine serum, with antibiotic concentrations as described above. After 12 days there was total cell death in the untransfected control plates (SHSY5Y subjected to Zeocin alone, Blasticidin alone, and both together),

whereas small colonies were growing in the transfected plates. Successful colonies were picked using a Pasteur pipette and grown in 12 well plates for 4-6 weeks at 37^{o}C in a 5% CO_2 humidified incubator. Selective media was replaced every 3-4 days.

36 colonies were grown, of which 10 were tested for transgene expression. Testing was performed by induction for 24 hours with 1μg/ml tetracycline, then Western blotting as described in 2.3.4. Two successful clones were obtained, and the clone with stronger expression chosen for further work. Successful clones were sequenced and the correct sequence confirmed. Christoph Grünewald confirmed DISC1 mRNA overexpression in the stronger expressing clone by qPCR on the iCycler (BioRad) to a level of 5.07 (+/- 0.21, n=3) compared to uninduced cells (1.1 +/-0.28, n=3). Protein overexpression was visualised by Western blot.

2.2.6 DISC1 knockdown cell lines

Fumiaki Ogawa kindly donated his DISC1 knock down SHSY5Y cell lines for my work (paper in submission). Briefly, for reference, shRNA constructs were cloned into linearised pENTR^{TM}/H1/TO using the Block-iT U6 system (Invitrogen). The shRNA constructs used to generate these cell lines can be found in Table 2.2. Stable cell lines generated from SHSY5Y as described in 2.2.5.2 using 50μg/ml Zeocin selection. T0 was a control line generated from a scrambled shRNA construct, shRNA for T1 was directed against the 3'UTR region, and for T2 against exon 2. Knock down of the DISC1 L isoform mRNA was confirmed by Christoph Grunewald using qPCR on the iCycler (Biorad) to levels of 0.004 (+/-0.003) and 0.002 (0.002) in T1 and T2 cells respectively, in comparison to the T0 scramble control (1 +/-0, n=3).

Cell line	Top strand Oligo	Bottom strand Oligo	Target
T0	CACCggatatcatgcaagtattaCGAAtaatacttgcatgatatccC	AAAAgggatatcatgcaagtattaTTCGtaatacttgcatgatatcc	Scrambled control
T1	CACCggatttgagaatagtttcaCGAAtgaaactattctcaaatccC	AAAAgggatttgagaatagtttcaTTCGtgaaactattctcaaatcc	3' UTR of DISC1
T2	CACCgcgtgacatgcattctttaCGAAtaaagaatgcatgtcacgcC	AAAAggcgtgacatgcattctttaTTCGtaaagaatgcatgtcacgc	Exon 2 of DISC1

Table 2.2: shRNA constructs used by Fumiaki Ogawa to generate stable cell lines with constitutive knock down of DISC1.

Name in text	Specificity	Species	Source	Conditions	Notes
Anti-Flag	Flag exogenous tag	Rabbit	Sigma	x10,000	
Anti-myc	c-myc exogenous tag	Mouse	Santa Cruz Biotechnology	x500	
D6692	DRD1	Rabbit	Sigma	x200	
MAB363	NR1	Mouse	Chemicon	x500	
PDE4B (MH)	Pan PDE4B	Sheep	Miles Houslay[1]	x1000	
PDE4D (MH)	Pan PDE4D	Sheep	Miles Houslay[1]	x1000	
αDISC1	DISC1	Rabbit	Professor Akiyama[2]	x2000	
Donkey anti sheep	Sheep Ig	Donkey	Invitrogen	x800	Secondary, fluorescent (594nm)
Goat anti mouse	Mouse Ig	Goat	Invitrogen	x500	Secondary, fluorescent (594nm)
Goat anti rabbit	Rabbit Ig	Goat	Invitrogen	x500	Secondary, fluorescent (488nm)
Goat anti rabbit	Rabbit Ig	Goat	Invitrogen	x800	Secondary, fluorescent (594nm)

Table 2.3A: A list of all antibodies used for immunocytochemistry in this thesis. Incubation times were 1 hour for all antibodies, primary and secondary. [1]Miles Houslay directs the Molecular Pharmacology Group, University of Glasgow. [2]Professor Akiyama heads his own group in the Laboratory of Genetic and Molecular Information, University of Tokyo.

Name in text	Specificity	Species	Source	Conditions	Notes
GAPDH	36 kDa GAPDH	Mouse	Chemicon	x80000 O/N	
PDE4B (MH)	Pan PDE4B	Sheep	Miles Houslay[1]	x5000 O/N	
PDE4D (MH)	Pan PDE4D	Sheep	Miles Houslay[1]	x5000 O/N	
Zymed C-term	DISC1 C terminal	Rabbit	Zymed	x200 O/N	
αDISC1	DISC1	Rabbit	Professor Akiyama[2]	x10000 O/N	
Donkey anti mouse	Mouse Ig	Donkey	Dako	x80000 30 mins	HRP conjugated secondary
Donkey anti sheep	Sheep Ig	Donkey	Dako	x2000 20 mins	HRP conjugated secondary
Swine anti rabbit	Rabbit Ig	Swine	Dako	x3000 20 mins	HRP conjugated secondary

Figure 2.3B: A list of all antibodies used for Western blotting in this thesis. O/N refers to overnight incubation with the primary antibody on a shaker at 4°C. Primary antibodies were diluted in blocking buffer, secondary antibodies diluted in wash buffer. [1]Miles Houslay directs the Molecular Pharmacology Group, University of Glasgow. [2]Professor Akiyama heads his own group in the Laboratory of Genetic and Molecular Information, University of Tokyo.

2.3 Protein Related Methods

2.3.1 Antibodies

Various antibodies were used to perform immunocytochemistry and a western blot during this project. Details of the antibodies and conditions used can be found in Table 2.4.

2.3.2 Cell and Primary Neuron lysate preparation

To produce cell lysates for the Western blot and PDE assays, SHSY5Y or HEK cells were grown to approximately 80-95% confluency in T25 cell culture flasks. Primary neuron cultures were grown in 6 well plates at a density of $1.5x10^6$ neurons per well. Cells were treated (either drug treatment or transfection), and the media aspirated off. Cells were then rinsed once with ice cold PBS, and the appropriate amount of lysis buffer added. 200µl of RIPA buffer was used per T25 for Western blotting. 200µl of 3T3 buffer was used for 3T3 assays. For neurons, 60µl of 3T3 buffer was added per well. Lysis buffer was well distributed across the surface by rolling, then a disposable cell scraper used to ensure detachment of all cells into the buffer. The lysate was then pipetted into labelled eppendorf tubes, and spun at 25rpm at 4^oC for 1 hour on a rotary wheel. Lysates were then centrifuged at 13,000 rpm for 60 minutes in a desk top centrifuge (Biofuge Fresco, Heraeus Instruments), and the supernatant removed to a fresh eppendorf. Lysates were stored at -80^oC until use.

2.3.3 Determining protein concentration of cell lysates

The concentration of total protein in cell lysates was measured using the BioRad protein concentration assay. Six concentrations of the BSA Protein Standard were prepared at 0, 0.2, 0.4, 0.6, 0.8 and 1mg/ml. Lysates were diluted ten times by adding 2.5µl of lysate to 22.5µl dH_2O in triplicate. To each of these preparations, 125µl of Reagent A and 1ml of Reagent B were added, and left to develop for up to 30 minutes at room temperature. The contents of each tube were removed to disposable cuvettes and read at an absorbance of 750nm using an Ultrospec 3000 Optical Reader (Pharmacia Biotech). The results for the protein standards were plotted in an excel spreadsheet to generate a

line graph. The equation for this line was used to determine the concentration of the cell lysates.

2.3.4 Western blotting

Western blotting allows the protein present in a crude lysate to be separated on a gel by virtue of their difference in size, in this case enabling detection of overexpressed DISC1 on SHSY5Y inducible cell lines.

20µg of RIPA lysed protein samples were diluted in dH_2O to 2µg/µl and mixed 1:1 with protein sample buffer, and 1:10 with DTT, to solubilise protein and reduce disulphide bonding respectively. Samples were then boiled for 5 minutes to denature protein, and disrupt protein-protein interactions. 15µl of each boiled mixture was loaded, along with a standardised protein size ladder (Precision Plus Protein All Blue Standard, BioRad) onto 7% Invitrogen gels (to detect DISC1 at 100kDa). Samples were electrophoresed at 150V for 60-90 minutes, in NuPage Tris Acetate SDS Running Buffer (Invitrogen) using an Xcell Surelock Electrophoresis Cell connected to a Power Pac 3000 (BioRad). Before transferring, an Invitrolon PVDF membranes (Invitrogen) was soaked in methanol then NuPage Transfer Buffer. Five sponges were also soaked in the transfer buffer. The gel containing the separated protein was then cracked open, and loaded into an XCell II Blot Module as follows: 3 x soaked sponges, card, gel, membrane, card, 2 x soaked sponges. The Blot Module was then filled with transfer buffer and connected to the PowerPac3000 at 30V for 60 minutes. The module was cooled by the addition of cold water to the exterior box. Ponceau staining confirmed protein transference onto the membrane, which was then blocked for one hour at room temperature on a rocker.

2.3.4.1 Immunostaining of Western blot

Post blocking, the primary antibody was added at the appropriate concentration to blocking buffer (see Table 2.3), and incubated overnight at 4^oC. Membranes were washed with wash buffer (2 quick washes, 1x15 minutes, 3x5 minutes while being

rocked at less than 35rpm), then stained with secondary antibody diluted in wash buffer for 20-30 minutes. The washing process was repeated before detection using ECL Plus Western blotting detection reagents (Amersham Biosciences). After 5 minutes of developing, ECL Plus was blotted from the membrane, and the membranes exposed to photosensitive paper (Scientific Laboratory Supplies) for 2-10 minutes. Film was developed using a Hyperprocessor (Amersham Life Sciences).

2.3.5 Immunocytochemistry

Immunocytochemistry (ICC) can be used to visualise the localisation of endogenous proteins or overexpressed tagged protein within cells. Primary antibodies directed against the protein of interest or the exogenous tag are incubated onto cells fixed to glass coverslips. Secondary antibodies can be attached to the primaries by virtue of the animal it is raised in, and these secondaries are connected to a fluorescent tag that can be visualised using a fluorescence or confocal microscope.

2.3.5.1 Fixing and immunostaining of cells for ICC

Before ICC can be performed, cellular processes must be halted, and protein epitopes exposed to antibodies by virtue of a process called fixing. Cells were grown on glass coverslips to around 75% confluency, to enable better visualisation of individual cells. Media was aspirated from the cells, which were then rinsed twice with ice cold PBS and fixed with chilled methanol for 10 minutes. Fixed cells were then washed three times with cold PBS.

Cover slips were blocked for 20 minutes at room temperature with 3% BSA in PBS. The wells were emptied, then primary antibodies diluted in 3% BSA/PBS were added for 1 hour. If two antibodies are being used, it is important to note that they must have been derived from different animals, in order to use two different secondary antibodies. Cells were rocked gently (less than 30rpm) throughout washes and incubation with antibodies.

Cover slips were rinsed with PBS (2x quick washes, 3x5minutes) then secondary antibodies diluted in 3% BSA/PBS were applied for one hour. As the secondary antibodies are fluorescent, the dish was wrapped in foil during this incubation. The washing process was repeated, before inverting the cover slips to mount them onto slides with Mowiol plus 250ng/µl DAPI. Slides were stored in the dark at 4^{o}C before visualisation using either a Zeiss Axioskop 2 microscope or more often, a Zeiss Laser Scanning Microscope 510 with LSM510 bespoke software.

2.3.5.2 Confocal Microscopy

Confocal microscopy enables more accurate analysis of colocalisation of immunostaining, by using lasers of the appropriate wavelength to image the signal from the fluorescent dyes in a single Z plane. Images can be taken at Z planes throughout the fixed sample, to give a cross sectional view of the cells being imaged. Confocal microscopy was performed on a Zeiss LSM510 Confocal Microscope with LSM510 bespoke software. As this particular microscope had no laser suitable for imaging DAPI staining, a fluorescent image of nuclear staining was also obtained with each confocal image. Single colour images and merged pseudo DAPI images were produced on IPLab software.

Colocalisation of two signals was further examined by the use of the Image Correlation Analysis plugin for ImageJ (Li et al., 2004). After background correction, this plugin generates a series of statistics relative to colocalisation. These are dealt with in great detail in the aforementioned reference, but basically consist of the Pearson correlation coefficient, (where -1 represents exclusion, 0 random localisation, and 1 complete correlation), The Mander's overlap coefficient (0 represents low colocalisation, 1 represents high colocalisation) and the Intensity Correlation Quotient (ICQ). The ICQ is the most complex of these measures, and takes into account the variation in intensities of each signal to produce a number between -0.5 and 0.5, where 0 equals random staining, negative figures show segregated staining and positive figures dependent staining.

2.4 Molecular Biology Methods

2.4.1 PCR Primers

Reference sequences of mouse cAMP PDEs and human PDE4s were identified from NCBI Entrez Gene and used to design exon boundary crossing primers using the NCBI Primer-BLAST designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). This relatively new tool combines the Ref Seq data on exon structure, Primer3 functionality for matching melting points and stipulating PCR product size, and the NCBI BLAST (Basic Local Alignment Search Tool) function to ensure specificity of primers. A complete list of all oligonucleotide primers used for PCR in this thesis can be seen in Table 2.4A-B. Primers were supplied by Sigma custom primer systems.

2.4.2 PCR Reactions

PCR was used as a tool to amplify untagged DISC1 for insertion into an expression plasmid, and to detect the expression of the various isoforms of PDE present in cDNA from whole mouse brain and primary neurons. Finally, quantitative real time PCR was used to detect fold changes in PDE4 expression in SHSY5Y cell lines overexpressing DISC1 and with constitutive DISC1 knockdown. Different versions of the basic PCR reaction were used for each of these functions. Basic PCR reactions were performed on a PTC-225 Peltier Thermal Cycler (MJ Research). Quantitative real time PCR was performed on the BioRad iCycler.

2.4.2.1 PfU Ultra Fusion Polymerase

To amplify DISC1 as in insert while introducing novel restriction sites, a high fidelity polymerase was required. The reaction was set up as follows:

1.0µl	5ng/µl DNA plasmid template
2.5µl	20pmols/µl 5' primer
2.5µl	20pmols/µl 3' primer
5.0µl	10x reaction buffer
1.25 µl	10mM dNTPs
Add dH_2O to 49µl	
1µl	PfU Ultra Fusion Polymerase (Stratagene)

Reaction conditions were as follows:

1. 95°C 2 minutes
2. 95°C 30 seconds
3. 60°C 30 seconds
4. 72°C 45 seconds
5. Repeat steps 2-4 an additional 29 times
6. 72°C 10 minutes
7. 4°C Holding temperature

2.4.2.2 Standard PCR Reaction

To detect the presence of PDE isoforms in cDNA, a standard PCR reaction was used.

2.0µl	10x PCR buffer
0.6µl	5mM dNTPs
0.25µl	5µM 5' primer
0.25µl	5µM 3' primer
2µl	cDNA template
Up to 17.8µl with dH_2O	
0.2µl	In house produced Taq

Reaction conditions were as follows:

1. 95°C 2 minutes
2. 95°C 30 seconds
3. 65°C 30 seconds
4. 72°C 30 seconds
5. Repeat steps 2-4 an additional 29/34 times
6. 72°C 10 minutes
7. 4°C Holding temperature

Name	Direction	Sequence	Purpose
WT DISC1 5'	5'	GATCGAATTCGCCGCCATGCCAGGCGGGGGTCCTCAGGGC	Cloning of DISC1 with a 5' EcoRI site
WT DISC1 3'	3'	GATCGCGGCCGCTCAGGCTTGTGCTTCGTGGACACC	Cloning of DISC1 with a 3' NotI site
HPan PDE4A fwd	5'	CCAGAACCTCAGCAAGCGCCA	Detection of PDE4 expression changes
HPan PDE4A rev	3'	TCCGGAGGACCTGGATGCGG	Detection of PDE4 expression changes
HPan PDE4B fwd	5'	CCGATCGCATTCAGGTCCTTCGC	Detection of PDE4 expression changes
HPan PDE4B rev	3'	TTTCCATTCCCCTCTCCCGCT	Detection of PDE4 expression changes
HPan PDE4C fwd	5'	GACGCCCTTTGCCCAGGTCC	Detection of PDE4 expression changes
HPan PDE4C rev	3'	CCAGCTTCTGCCCCGTGTCC	Detection of PDE4 expression changes
HPan PDE4D fwd	5'	TGCTCAGGTCTTGGCCAGTCTGC	Detection of PDE4 expression changes
HPan PDE4D rev	3'	TCCTCCAGGGTCTCGCTGGC	Detection of PDE4 expression changes
GAPDH fwd	5'	TCAAGATCATCAGCAATGCC	Detection of "Housekeeping" genes in qPCR[1]
GAPDH rev	3'	ATCCACAGTCTTCTGGGTGG	Detection of "Housekeeping" genes in qPCR[1]
RPLPO fwd	5'	AATGGCAGCATCTACAACCC	Detection of "Housekeeping" genes in qPCR[1]
RPLPO rev	3'	CCGTTGATGATAGAATGGGG	Detection of "Housekeeping" genes in qPCR[1]
PPIA fwd	5'	AGACTGAGTGGTTGGATGGC	Detection of "Housekeeping" genes in qPCR[1]
PPIA rev	3'	TCGAGTTGTCCACAGTCAGC	Detection of "Housekeeping" genes in qPCR[1]
B2M fwd	5'	AAAGATGAGTATGCCTGCCG	Detection of "Housekeeping" genes in qPCR[1]
B2M rev	3'	AGCAAGCAAGCAGAATTTGG	Detection of "Housekeeping" genes in qPCR[1]

Table 2.4A: A table showing the human PCR primers employed during this project. Primers based on human sequence information were used for cloning of DISC1 for insertion into the pcDNA4/TO vector, detection of PDE4 isoform expresson changes in SHSY5Y based DISC1 knockdown and over expressing lines, and detection of stable “reference” genes in qPCR. 1 PCR Primers for reference genes were designed and kindly supplied by Christoph Grünewald.

Name	Direction	Sequence	Purpose
Pan PDE2A fwd	5'	GGGCTGGGACCTTCGGACCT	Detection of PDE isoforms
Pan PDE2A rev	3'	GCAGGGCTACCAGAGCGTGC	Detection of PDE isoforms
Pan PDE3A fwd	5'	CGGCTCAGCAAGCGACTCCG	Detection of PDE isoforms
Pan PDE3A rev	3'	ATAGCACCGCCTGAGGGGCA	Detection of PDE isoforms
Pan PDE3B fwd	5'	AGCTCTGGAGCCTCATCGTTGCT	Detection of PDE isoforms
Pan PDE3B rev	3'	GCTGGGCAAAGCACCAGCTCT	Detection of PDE isoforms
Pan PDE4A fwd	5'	CACGCCTGCACTGGATGCCG	Detection of PDE isoforms
Pan PDE4A rev	3'	CGCTGGCGCTTGCTGAGGT	Detection of PDE isoforms
Pan PDE4B fwd	5'	AGCCAAGGAGCTGGAAGACCTGA	Detection of PDE isoforms
Pan PDE4B rev	3'	CTGGGCCACGTCAGCAGCAT	Detection of PDE isoforms
Pan PDE4C fwd	5'	CGCATCCAGCAGGGTGACCG	Detection of PDE isoforms
Pan PDE4C rev	3'	TGTGTGGAGGGCTGCAGGGA	Detection of PDE isoforms
Pan PDE4D fwd	5'	TGGGGCCTCCACGTTTTCCG	Detection of PDE isoforms
Pan PDE4D rev	3'	ACACAGCCTCCAAAGCGGGTG	Detection of PDE isoforms
Pan PDE7A fwd	5'	ATTGCAGCCGCCACTCACGA	Detection of PDE isoforms
Pan PDE7A rev	3'	TGAGCCTCCATCTCTTGCCTGCT	Detection of PDE isoforms
Pan PDE7B fwd	5'	ACAAGCAAGGCACATGCTCTCCA	Detection of PDE isoforms
Pan PDE7B rev	3'	GGTGACGTCGGCTGCGTGAA	Detection of PDE isoforms
Pan PDE8A fwd	5'	AGCCCAGGAGAGTGAGGAACCTG	Detection of PDE isoforms
Pan PDE8A rev	3'	CACGGGCATCACCACAGGCA	Detection of PDE isoforms
Pan PDE8B fwd	5'	TGGCAGCTGAGAGTGAGGGCA	Detection of PDE isoforms
Pan PDE8B rev	3'	GCATCACCACAGGCAGCCCC	Detection of PDE isoforms
Pan PDE10A fwd	5'	GTCCCTCAAGGGCAGCTCGG	Detection of PDE isoforms
Pan PDE10A rev	3'	TCAGGTGTGCTCTTGCTAGGCG	Detection of PDE isoforms

Table 2.4B: A table showing the mouse PCR primers used during this project. Primers based on mouse sequence information were used for detecting cAMP PDE isoforms in cDNA prepared from whole mouse brain and cultured primary neurons.

2.4.3 Quantitative Real Time PCR

Quantitative RT PCR was used to assess potential changes in the expression of the PDE4 isoforms in TRTODISC1 after 24 hours tetracycline treatment, and to compare the constitutive knock down line T1 with its T0 control line. Primers were designed as detailed in 1.4.1, and tested on one cDNA sample and a no reverse transcriptase control. Primer specificity was confirmed for hPDE4A, hPDE4B and hPDE4D, using the PCR protocol below. No expression was detected for hPDE4C, as was predicted from the literature. Standard dilution curves were performed for the three successful primers, using consecutive factor of 10 dilutions of cDNA from undiluted to 1:1000. Successful standard curves were obtained, and final experiments performed using 3 sets of cDNA kindly supplied by Christoph Grünewald. 2 technical replicates of these samples were performed.

2.4.3.1 Basic Quantitative PCR Reaction

Where large scale experiments were performed, two master mixes were prepared. The first of these contained SybrGreen, cDNA and dH_2O. The second contained primer pairs diluted in dH_2O.

10µl	SybrGreen (BioRad)
1µl	cDNA diluted 1:10
1µl	10µM forward primer
1µl	10µM reverse primer
7µl	dH_2O

Reaction conditions were as follows

1. 95^oC 2 minutes
2. 95^oC 30 seconds
3. 60^oC 30 seconds

4. 80°C 30 seconds
5. Repeat steps 2-4 an additional 35 times
6. 72°C 10 minutes
7. 4°C Holding temperature

Analysis of fold changes was performed in Excel using values obtained from MyIQ bespoke software (BioRad).

2.4.4 Sequencing

Sequencing was used to confirm the correct sequence of the DISC1 insert post cloning. DISC1 sequencing primers were kindly designed and provided by Shaun Mackie, who ordered them from Invitrogen custom primer service. All sequencing was performed in 96 well plates.

2.4.4.1 Big Dye pre-sequencing PCR

The Big Dye Terminator Ready Reaction Mix v3.1 system was used. Sequencing reactions were set up as follows:

1.5µl	5 x Sequencing Buffer (Applied Biosystems)
1.0µl	Big Dye 3.1 (Applied Biosystems)
~1µl	Template (at least 0.5µg DNA)
0.5µl	60ng/µl primer

Reactions were then processed using the Big Dye 3.1 program as follows:

1. 96°C 1 minutes
2. 96°C 10 seconds
3. 50°C 5 seconds
4. 60°C 4 minutes

5. Repeat steps 2-4 an additional 24 times
6. 4°C Holding temperature

2.4.5 Sequencing plasmids

Sequencing was performed on the DISC1 insert post cloning into pcDNA4/TO, to ensure no mutations had been introduced in the process. Firstly, plasmid DNA was used in pre sequencing reactions described in 2.4.5.1**,** using a series of primers corresponding to adjacent and overlapping regions of DISC1. These primers are detailed in Table 2.5. This enables sequencing of the whole open reading frame.

The product was then precipitated via a series of steps. All reagents used were sterile. 5µl of 125mM EDTA (Sigma), then 60µl of 95% ethanol (Fisher) was added to each reaction product. Plates were briefly inverted and left to stand for 15 minutes, before spinning for 30 minutes at 3000rpm on a Jouan CR422 centrifuge. Post centrifugation, ethanol was removed via tapping and pulse spinning the plate upside down. 60µl of 70% ethanol was added to each well, and the plate spun at 3000rpm for 15 minutes. Again, ethanol was removed via tapping and pulse spinning. Plates were then air dried for 30 minutes in the dark, taken to the sequencing facility for processing, and held at -20°C prior to analysis.

The nucleic acid sequence of the precipitated product was determined by Agnes Gallacher at the Medical Research Council Human Genetics Unit. Results were viewed using Chromas Lite version 2.01 (Technelysium Pty Ltd).

Name	Direction	Sequence	Purpose
M150S	5'	ATGGATAGTTCTGAGACCCT	Sequencing of DISC1 from pcDNA4/TO[1]
S301S	5'	TCCTCCAGTTCTCTGGATCC	Sequencing of DISC1 from pcDNA4/TO[1]
S451S	5'	TCCATCACGAGACGAGACTG	Sequencing of DISC1 from pcDNA4/TO[1]
K551S	5'	AAATCCCTCAACTTGTCACT	Sequencing of DISC1 from pcDNA4/TO[1]
A180AS	3'	TGCTGATGGCAGAGAGCCTG	Sequencing of DISC1 from pcDNA4/TO[1]
I560AS	3'	GATTTCTTTAAGTGACAAGT	Sequencing of DISC1 from pcDNA4/TO[1]
CMV Fwd	5'	CGCAAATGGGCGGTAGGCGTG	Sequencing of DISC1 from pcDNA4/TO[2]
BGH Reverse	3'	TAGAAGGCACAGTCGAGG	Sequencing of DISC1 from pcDNA4/TO[2]

Table 2.5: a list of primers used to sequence DISC1 post cloning. [1]Primers designed and supplied by Shaun Mackie. [2]Primers supplied as part of Invitrogen TRex cloning kit.

2.4.6 Purification of PCR products

Prior to ligation into the expression plasmid, the PCR amplified DISC1 insert was purified to remove elements of the PCR reaction mix which may contaminate further reactions. This was performed using a QIAquick PCR purification kit (Qiagen). The spin protocol was followed, as per the manufacturer's instructions.

2.4.7 Making and Running Agarose DNA gels

Agarose gels were used to assess the size of DNA products obtained by PCR, and the efficiency of restriction enzyme digestion. Gels were prepared by adding the appropriate concentration (usually between 1-2%) of agarose (Invitrogen) to 0.5x TBE buffer, then gently heated. Sybr Safe DNA Gel Stain (BioRad) was added at a concentration of 5µl per 100ml agarose solution, and the gel poured into a tray with the appropriate number of combs. Once the gel had set, combs were removed, and the gel placed in an electrophoresis tank filled with 0.5x TBE buffer (Bioscience Service).

The DNA samples were then loaded into the wells of the gel, alongside an appropriately sized DNA ladder. Gels were run at a voltage of less than 100mV for up to two hours, then viewed with UV light in a Uvidoc light box (Uvitec).

2.4.8 RNA extraction

RNA was extracted from whole mouse brain and cultured primary neurons using the Qiagen RNeasy Mini Kit. Cultured primary neurons were lysed as previously described with RLT buffer (Qiagen kit component). Whole mouse brain was dissected from an adult CD1 mouse, placed immediately in RNA later (Applied Biosystems) and stored at 4^{o}C. Before RNA extraction, the brain was homogenised by chopping into small sections and placing into Precellys 1.4mm Ceramic homogeniser tubes (Peqlab) with RLT buffer. Samples were then homogenised in the Precellys 24 homogeniser (Stretton scientific), and applied to the RNeasy protocol as per the Manufacturer's instructions. In all cases the additional step of on column DNase digestion was performed.

2.4.9 cDNA Synthesis

Before cDNA synthesis was performed, an extra DNA clean up was performed using Turbo DNAfree (Ambion) according to the manufacturer's instructions. Depending on the yield of RNA from extraction, two different methods of cDNA synthesis from RNA were employed.

2.4.9.1 First Strand (AMV) cDNA Synthesis Kit (Roche)

This kit was used when a high yield of RNA had been obtained, as it requires a smaller volume of RNA. RNA samples were diluted in dH_2O to a concentration of 2µg RNA per 16.4µl (half this for the no RT control). Samples were denatured for 15 minutes at 65^{o}C, then placed on ice for 5 minutes. During this process, two mixes were prepared. All reagents come from the First Strand cDNA synthesis kit.

Mix 1

4µl	10x buffer
8µl	25mM $MgCl_2$
4µl	dNTPs
4µl	random primers

2µl RNase inhibitor

1.6µl AMV Reverse Transcriptase

Mix 2 (no RT control)

2µl 10x buffer

4µl 25mM $MgCl_2$

2µl dNTPs

2µl random primers

1µl RNase inhibitor

After denaturing, 23.6µl of Mix 1 was added to each 2µg RNA sample, and 11µl of mix 2 added to each 1µg no RT control. A no RNA control was also employed. All samples were then incubated at 42°C for 1 hour, then 95°C for 10 minutes to denature the enzymes. Samples were cooled to 4°C, then stored at -20°C.

2.4.9.2 Transcriptor First Strand cDNA Synthesis (Roche)

This method allows for a larger volume of RNA if RNA yield was low. Again, all reagents used are from the Roche kit of the same name. 2µg of each RNA sample was diluted into 22µl dH_2O, and 1µg into 11.5µl for the no RT controls. 4µl random primers were added to each sample (2µl for the no RT controls) and the mix denatured for 10 minutes at 65°C, then cooled to 10°C.

Once again, two mixes were prepared:

Mix 1

8µl 5x buffer

1µl RNase inhibitor

4µl dNTPs

1µl Transcriptor Reverse Transcriptase

Mix 2

4µl	5x buffer
0.5µl	RNase inhibitor
2µl	dNTPs

After denaturing, 14µl of Mix 1 was added to each 2µg RNA sample, and 6.5µl of mix 2 added to each 1µg no RT control. A no RNA control was also employed. All samples were then incubated at 25°C for 10 minutes, 55°C for 30 minutes, then 85°C for 5 minutes to denature the enzymes. Samples were stored at -20°C.

2.4.10 Transformation of E coli with plasmid constructs

Transformation of E coli allows propagation of DNA plasmids for further use. For this purpose a heat shock protocol was used, with JM109 Competent E Coli (Promega). First, 100µl of JM109 cells per reaction were thawed slowly on ice. Once thawed, the cells were mixed gently by flicking, and 50ng of a DNA plasmid diluted into 1µl of dH_2O was added to the mixture. The whole mixture was incubated on ice for 30 minutes, before being heat shocked by removal to a water bath at 42°C for 45 seconds. After the shock, cells are removed back to ice for 2 minutes. 900µl of room temperature L Broth was added to the cells, which were then shaken at 200rpm at 37°C for 45 minutes in an Innova incubator shaker (New Brunswick Scientific). After 45 minutes, 100-200µl of the bacterial culture was spread evenly onto pre poured Agar plates containing the appropriate antibiotic using a sterile disposable spreader. Plates were incubated upside down at 37°C with 5% CO_2 overnight (Plus II incubator, Gallencamp), or until colonies were visible.

2.4.11 Purification of plasmid constructs from E coli

In order to purify DNA from transformed cultures, single clones must be selected from the agar plate and amplified further. To do this, single colonies are picked from the plate using a disposable sterile loop. This loop was placed in 5ml L-Broth containing

the appropriate antibiotic and shaken at 200rpm at 37°C with 5% CO_2 for 6-8 hours to assure growth. These mini cultures can be pelleted by centrifugation at 13,000rpm for 1 minute, and the plasmids harvested by using a Spin Miniprep kit (Qiagen). This method of purification is quick, easy, and is ideal for analysing plasmids post cloning.

If larger amounts of plasmid were required, for transfection for example, these mini cultures were then transferred to 400ml of L-Broth with the appropriate antibiotic, and subjected to the same incubation conditions overnight. The next morning the bacterial pellet was harvested by centrifuging at 5000rpm, 4°C for 10 minutes in an Avanti J-201 centrifuge (Beckman Coulter), and subjected to purification using the Qiagen Plasmid Maxiprep Kit as per the manufacturer's instructions. Purified DNA from both methods was suspended in dH_2O and stored at 4°C for short term storage, or -20°C for longer term.

2.4.12 Measuring DNA/RNA concentration

In order to determine the concentration of a purified plasmid or extracted DNA, the Nanodrop 2000 spectrophotometer (ThermoScientific) was used. 1µl of nucleic samples were loaded onto the pedestal, the correct option selected in the bespoke software, and an absorbance reading is taken automatically at the correct wavelength. The software produces a graph and two ratios which give information about the quality of the nucleic acid tested. 260/280nm ratios of approximately 1.8 and 2.0 are generally considered pure for DNA and RNA respectively. The 260/230 ratio should be between 2-2.2, and a lower reading may indicate the presence of carbohydrate or phenol in the preparation.

2.4.13 Cloning techniques

2.4.13.1 Restriction Digests

Restriction enzyme digestion was used to cleave plasmid DNA at the desired cloning sites. Restriction enzymes are supplied with their appropriate buffer, in this case they were EcoRI and NotI (Both buffer H, Roche). 3µg of plasmid DNA, and 40µl of post

clean up insert DNA was diluted in 1x buffer H and dH_2O, and 3 or 4µl of restriction enzyme added to the mix respectively. The reaction was incubated for 1 hour at 37°C. The digested product plasmid was run on a gel to confirm that full digestion had occurred.

2.4.13.2 Phenol-chloroform extraction and DNA precipitation

In order to use the digested DNA in a ligation reaction, contaminating proteins remaining from the digest must first be extracted. 100µl of phenol/choloroform/isoamyl alcohol mix was added to each DNA digest. The mixture was vortexed then spun at 13,000rpm for two minutes to separate the phases. 40µl of the aqueous phase was removed to a fresh eppendorf tube.

The remaining mixture underwent back extraction by addition of 40µl of TE buffer (Qiagen), then repeating the vortex and centrifuge procedure. A further 80µl of the aqueous phase was removed, and added to the fresh eppendorf containing the 40µl fraction. 1.2µl 3M sodium acetate (Fisher), 240µl absolute ethanol and 2µl of glycogen (Promega) was added to the eppendorf, and this solution frozen at -20°C for at least 30 minutes. After this time, the solution was thawed and spun at 13,000rpm on a desktop centrifuge for 30 minutes to produce a DNA pellet. The supernatant was discarded, the pellet resuspended in 70% ethanol, and re-centrifuged for 5 minutes at 13,000rpm. Again, the supernatant was discarded, and the pellet air dried for up to 15 minutes. Once dry, it was finally resuspended in 30µl dH_2O.

2.4.13.3 SAP Phosphatase Treatment

Following restriction digestion, extraction and DNA precipitation, the DNA vector was treated with shrimp alkaline phosphatase (USB Corporation), to remove phosphate groups and hence decrease the likelihood of self ligation of the vector. 10µl of the vector DNA was made up to 20µl with SAP buffer, and 1.5µl of the SAP enzyme added.

The reaction was incubated at 37°C for 30 minutes, and terminated by incubation at 70°C for 10 minutes to denature the SAP enzyme.

2.4.13.4 Ligation reaction

The final point in the subcloning procedure was ligation of the insert into the DNA vector, following restriction digestion, extraction, DNA precipitation and SAP phosphatase treatment of the vector. Ligation reactions were set up to 10µl, containing 1µl T4 ligase (Roche), 2µl vector and 5µl insert (with a 1:3 ratio of vector to insert) in T4 ligase buffer. Reactions were incubated at room temperature for at least four hours, and the resulting DNA transformed into JM109 competent E coli for amplification and testing.

2.5 Functional Assays

2.5.1 Phosphodiesterase activity assay

PDE Assays were performed to a protocol adapted from Marchmont and Houslay (Marchmont and Houslay, 1980). Cell and tissue lysates were prepared using 3T3 buffer plus Complete Protease Inhibitor cocktail tablets (Roche) and Phosphatase Inhibitor II (Calbiochem). Volumes used were 200µl per confluent T25 flask and 60µl per well of a 6 well plate. Lysates were prepared as described in section 2.3.2.

A standard amount of lysate protein (determined by concentration curve) was diluted in pH7.5 Tris solution (Fisher) to a total of 50µl. Generally 20µg protein was used per reaction for SHSY5Y lysate, and 15µg per reaction for primary cortical neuron lysate. 6 replicates of each sample were set up, half with the PDE4 inhibitor Rolipram (1µl of a 1mM stock solution, Sigma) to enable the PDE4 component of activity to be measured.

In the first stage of the two step assay, 50µl of the PDE assay substrate solution was added to the 50µl lysate solutions. Final reaction concentrations of PDE Assay buffer

components were therefore 5mM $MgCl_2$, 1μM cAMP, and 200,000 decays per minute arising from [^{3}H] cAMP. The reaction was vortexed, spun down on a desk top centrifuge, and incubated at 37^{o}C for 10 minutes. The reaction was then terminated by removal to a boiling water bath for two minutes. Tubes were then placed on ice for 15 minutes, before the addition of 25μl of Western Diamondback Snake Venom (Sigma). This mixture was incubated for a further 10 minutes, in which the [^{3}H] labelled cAMP hydrolysis product, 5'AMP is dephosphorylated to adenosine by the snake venom. The negatively charged non hydrolysed cAMP is then separated out of the reaction mixture by additional of 400μl of Dowex ion exchange resin (Sigma, made up to slurry 1:1:1 Dowex:water:absolute alcohol), left to stand for 15 minutes with inversion of the tubes every five minutes, followed by centrifugation at 14,000rpm for 3 minutes. 150μl of the supernatant was carefully removed and added to 1ml of Ecoscintillant fluid (AGTC BioProducts), the tubes inverted, and read on a LS6500 Multi Purpose scintillation counter (Beckman Coulter, Human Genetics Unit, Medical Research Council). Background counts were accounted for by use of a Tris buffer only control.

2.5.1.1 Protein concentration curves

To determine the appropriate amount of protein to utilise when PDE assaying different cell types, protein concentration curves were determined. A range of protein quantities were assayed as described earlier, and plotted on a graph. An amount of protein in the linear phase of the graph was chosen for maximising detection of changes. PDE activity values were calculated using the equation below:

PDE Activity = 2.61 x (value-blank/total counts in assay) $x10^{-11}x\ 10^{12}x$(1000/μg protein)

2.5.2 cAMP AlphaScreen (Perkin Elmer)

The basis of the Perkin Elmer AlphaScreen is a competition assay, where streptavidin coated donor beads bind to exogenous biotinylated cAMP, under low endogenous cAMP conditions. When acceptor beads conjugated to a cAMP antibody are added to a

solution containing these molecules, the antibody also binds the biotinylated cAMP, bringing the acceptor and donor beads into close proximity. On excitation by the EnVision microplateplatereader (Perkin Elmer) at 680nm, this proximity allows for the transfer of singlet oxygen between the donor and acceptor bead. This transfer results in fluorescence of the acceptor bead at 520-620nm, which can then be measured on the plate reader. When endogenous cAMP is increased, by Forskolin addition, for example, the endogenous cAMP displaces the exogenously biotinylated cAMP from the donor beads, increasing the distance between the donor and acceptor beads, and preventing the transfer of singlet oxygen. Fluorescence intensity at 520-620nm is decreased. To summarise, the higher the endogenous cAMP, the lower the fluorescence intensity. This is an important point to remember for interpretation of graphs.

2.5.2.1 cAMP Standard Curve Procedure

All initial AlphaScreen work was performed as described in the AlphaScreen manual. To briefly summarise, a standard cAMP solution was serially diluted in Control Buffer at pH7.4 to provide a concentration range of $5x10^{-6}$ to $5x10^{-11}$M cAMP with 11 assay points. A positive control with no cAMP (highest signal) was also prepared. The "detection mix" combining streptavidin coated donor beads and biotinylated cAMP was prepared fresh, and incubated in the dark for 30 minutes.

While this solution was incubating, the anti-cAMP acceptor bead solution was prepared. In triplicate, 5µl of the anti-cAMP acceptor bead mix was added to 12 rows of an OptiPlate-384 well plate (Perkin Elmer). This was followed by the addition of 5µl of each of the cAMP dilutions, sequentially to the plate. The plate was incubated in the dark at room temperature for 30 minutes. After 30 minutes 15µl of the detection mix were added to each of the wells in use, and the plate incubated in the dark at room temperature for 1 hour. The plate was then read on the EnVision Microplate Reader, and data plotted as a signal curve corresponding to the log concentration of cAMP.

2.5.2.2 Forskolin Dose-Response / Determination of the Optimal Cell Concentration

This protocol is slightly adapted from the one in the manual, as I chose to perform the assay on adherent cells. Two days prior to the assay a T_{25} flask of 95% confluent SHSY5Y cells was harvested. Cells were resuspended, counted on a haemocytometer, and spun at 1000rpm for 5 minutes on a centrifuge. Post centrifugation, the supernatant was removed, and the cells resuspended to a concentration of 10,000 cells/µl. Three further dilutions were then produced, according to the Perkin Elmer booklet, as reproduced in Table 2.6.

Cells/well (final)	10,000cells/µl solution (µl)	DMEM (µl)
10,000	50	200
3,000	15	240
1,000	5	245

Table 2.6: Table showing the dilution of cells performed to prepare SHSY5Y cells for the cell number optimisation assay.

5µl of the cell concentration solution was added in triplicate to 12 rows each of a Perkin Elmer OptiPlate-384 well plate. 50µL of warm DMEM with 10% FBS was then added to each well containing cells, and the plate incubated for two days at 37^{o}C with 5% CO_2.

To perform the cell number optimisation assay 40ml of Stimulation buffer containing IBMX was prepared to pH 7.4. Lysis buffer was also prepared to pH 7.4. A forskolin serial dilution series was prepared in stimulation buffer, amounting to 11 concentrations of forskolin ranging between $2x10^{-4}$ to $2x10^{-9}$M in ½ log intervals, and a no forskolin control. Detection mix was then prepared and incubated in the dark at room temperature for a minimum of 30 minutes. Finally, Acceptor bead mix was made.

The 384 well plate containing the cells was then rinsed twice carefully with stimulation buffer, and dried by tapping. The reagents were added to the wells. First added was 5µl of the Acceptor bead mix, followed by 5µl of the Forskolin dilutions (1 dilution per row of the assay), and included a cAMP standard control (5µM) and a no cells, no cAMP

positive control. This mixture was then incubated in the dark for 30 minutes at room temperature. After 30 minutes, 15µl of the Detection Mix was added to each well, and the plate incubated in the dark at room temperature for 4 hours. The plate was rocked for the first ten minutes to encourage full lysis of cells. After 4 hours the plate was read on the EnVision microplate reader.

2.5.2.3 Lithium Chloride cAMP Screen

To screen SHSY5Y cells for an effect of LiCl on cAMP concentration, an adapted version of the cell number optimisation assay with no IBMX in the stimulation buffer was performed. This enables detection of cAMP changes resulting from changes in PDE activity. For this experiment, a dilution series of LiCl (Sigma) in stimulation buffer from 0-20mM in 2mM intervals was prepared, along with a 20µm forskolin solution in stimulation buffer. 5µl of each LiCl dilution was added to 6 wells of the 384 well plate, containing 10,000 initial adherent cells per well. To half of these dilutions 5µl of stimulation buffer was added, and to the other half, 5µl of 20µM forskolin solution. After incubation for 30 minutes in the dark at room temperature, 15µl of Detection mix was added to each well, and the procedure followed as described in 2.5.2.2.

3 Assay work up and SHSY5Y as a model system

3.1 Introduction

In order to investigate the potential for DISC1 and chemical compounds to alter PDE4 activity, two factors must be considered. The first is the use of assays that are sufficiently sensitive and appropriate to measure cellular changes resulting from alterations in PDE4 activity, which are likely to be subtle. The second is the use of a suitable cellular system for PDE4 measurement which should be easy to manipulate, both genetically and chemically, while producing highly consistent and reproducible measurements. This chapter deals with the choice of assays, the work up of these assays, and the use of SHSY5Y human neuroblastoma cells as an appropriate model cellular system for measuring PDE4 activity.

3.2 Choosing an assay for PDE activity

There are two major routes available to an investigator wanting to assess the activity of a PDE enzyme. The first of these routes is to perform an investigation of protein activity. PDE activity can be assayed directly, through loading of cells with radiolabelled cAMP, but the majority of methods employed rely on use of *in vitro* purified enzyme (Skoumbourdis et al., 2008), or overexpression of the enzyme or related cellular components in a stable cell line system. The drawbacks of assaying purified enzyme separated from its cellular context are clear: only compounds directly affecting the enzyme itself will show as "hits," and no information is obtained about the membrane permeability of the compound. Overexpression of PDE or related reporter proteins in cell lines avoids these particular drawbacks, but can lead to aberrant long term regulation of the pathways being investigated. This leads to the second route of investigation, which infers PDE activity indirectly by measuring cellular cAMP fluctuations, often without the need for expression of exogenous protein. Each of these

routes offers a number of choices of assay with a variety of advantages and disadvantages, which I will briefly review in this section.

3.2.1 cAMP concentration/accumulation assays

Early methods to measure cAMP in cells relied on rather labour intensive processes of extraction, or labelling the ATP pool with tritium to measure radiolabelled cAMP. These methods provide highly accurate measures of whole cell cAMP levels, and formed the base for cAMP assays for many years, but have been surpassed by recent technology due to their lack of spatial and temporal resolution (reviewed by Willoughby and Cooper, 2008).

These early methods have since been adapted to increase assay throughput (reviewed Williams, 2004). To summarise briefly, there are a large number of commercially available cAMP accumulation assays on the market, which are generally based on the principle of competition between endogenous cAMP and exogenous cAMP labelled either fluorescently, radioactively, with bioluminescence or with enzyme donors. Usually these commercial assays require specific microtitre well plates and plate readers, thus making this a primary consideration in assay choice. The Perkin Elmer AlphaScreen is just one example of this technology, which uses luminescence proximity to produce a signal detectable by the plate reader (see Williams, 2004 for more commercially available options). These assays are generally more suitable for measurement of cAMP accumulation after GPCR activity, as sensitivity is increased if PDEs are blocked with a substance such as IBMX. These assays can usually be performed on live cells or pre prepared protein lysates, but generally rely on a cell lysis step before the final reading.

As Williams (2004) also details, the other main procedure for assaying cAMP relies on reporter gene constructs such as luciferase and β-galactosidase which contain cAMP responsive elements (CRE). Binding of cAMP to these elements results in a highly

sensitive measure of cAMP concentration within the cell. In fact, at very low levels of cAMP this approach may actually amplify the signal generated in a non linear fashion, falsely inflating the effect of a compound. As with any assay dependant on a genetic sensor, the main limitation is the creation and initial testing of a stable cell line, which can be time consuming.

In recent years there has been a vast explosion in the number of genetically encoded intracellular cAMP sensors available. These are reviewed in great detail in Willoughby and Cooper (2008), but are mostly founded on changes in activity of downstream proteins, such as FRET or BRET based cAMP dependant PKAs and "exchange protein directly activated by cAMP" (Epac) assays. An alternative to these are measurements made from cyclic nucleotide gated channels using calcium sensing dyes or single channel electrophysiology, but a major limitation of these sensors is that they can only sense changes occurring close to the plasma membrane (Willoughby and Cooper, 2008). These probes provide excellent information on the location and timing of cAMP fluxes, and some combinations of sensors which use ratiometric read outs from two wavelengths directly correlate to calcium or cAMP concentration, thus are suitable for miniaturisation. These systems have been used reliably in high throughput screens (Reinscheid et al., 2003, Jiang et al., 2007), but are complex and time consuming to set up and calibrate.

3.2.2 Lysate based PDE4 activity assay

Marchmont and Houslay (1980) took the approach of radiolabelled cAMP (discussed above) a step further by loading cell lysates with a known amount of tritiated cAMP, which was then broken down by endogenous PDE activity. The break down product, 5'AMP, is then dephosphorylated using snake venom, leaving an uncharged product which can be separated from negatively charged, uncleaved cAMP. This tritiated breakdown product can then be measured on a scintillation counter (Marchmont and Houslay, 1980, Figure 3.1). The advantage of this method is that it negates the need for

overexpression of cAMP pathway components, and so genetic and chemical manipulations can be performed without the confounding factor of pathway changes. The major disadvantage to this method is that it is not directly PDE isoform specific. Specific activity can be inferred from the use of replicates treated with isoform specific inhibitors, or is achieved via antibody mediated immunoprecipitation of specific isoforms (Millar et al., 2005b). The assay also has multiple steps, is labour intensive, and the need for multiple centrifugation steps limits throughput.

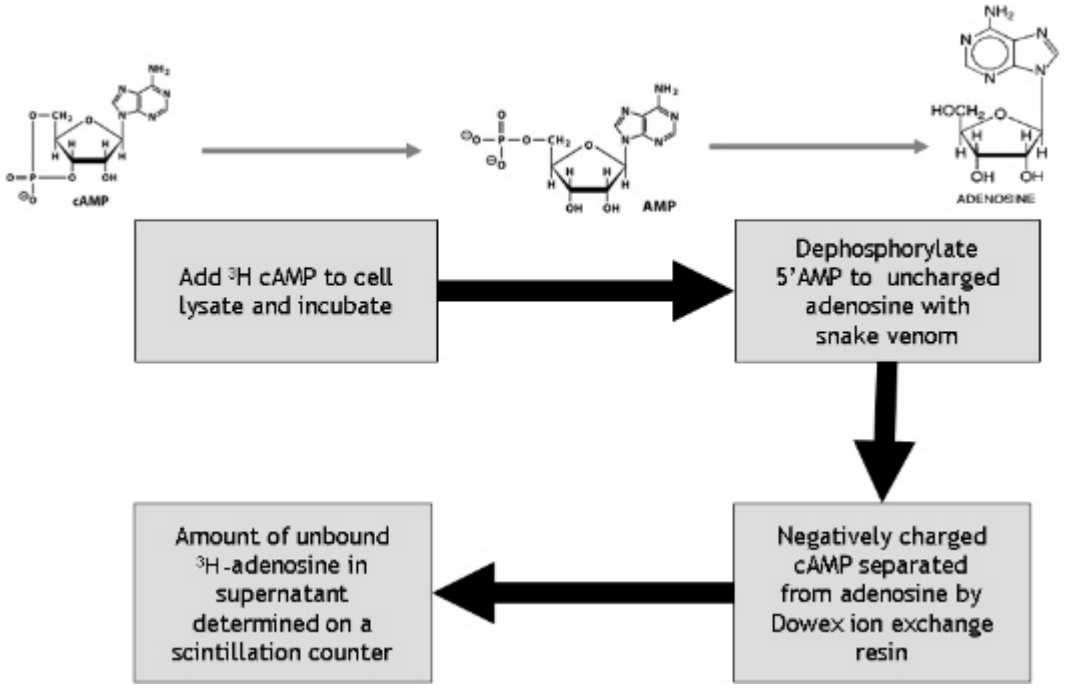


Figure 3.1 depicts the procedure adapted from Marchmont and Houslay for measuring PDE activity in cell lysates. Diagrams to show the alterations to the chemical structure and charge of cAMP from each step can be found above the experimental scheme. To make the assay PDE4 specific, six replicates are used in total, 3 treated with 10μM rolipram and 3 without. PDE4 activity can then be resolved by a simple subtraction of the activity remaining post rolipram treatment: the non-PDE4 component.

3.2.3 Live cell based assays for PDE4 activity

As exemplified by Bora *et al*, a common means of designing cell based assays for PDE activity relies on stable overexpression of a PDE isoform. For their assay, they transiently transfected stable HEK293 cell lines overexpressing PDE4 isoforms with a CRE based luciferase reporter (Bora et al., 2007). While this technique has the advantage of being able to assess PDE4 subtype specific effects of compounds, it relies on the constitutive overexpression of a protein that may significantly alter intracellular signalling pathways. Allen *et al* took a similar approach, transiently overexpressing PDE4A-D with a β-adrenergic receptor that can be stimulated with isoprenaline to elevate cAMP. PDE activity is obtained indirectly by measuring the amount of 5'AMP generated. This assay system was successfully used to assess potential new PDE4 inhibitors (Allen et al., 1999). It is clear that to avoid variability being introduced by variations in transient transfection rate, a very rigorous system of cell counting, timing, and reagent measurement must be employed. This adds an extra level of complexity to a screening procedure.

Titus *et al*, 2008 extended the approach of Rich *et al*, 2001, to use a HEK293 based stable cell line with a constitutively active adenylate cyclase coupled GPCR, co expressed with a cyclic nucleotide gated (CNG) cation channel. This method enables measurement of PDE activity in a 1536 well plate as a function of changes in calcium levels (Rich et al., 2001) or membrane potential using a membrane potential sensitive dye (Titus et al., 2008). PDE4 specificity was confirmed by Titus *et al* in a cell free PDE4 A1A assay, and 7 potential novel PDE4 inhibitors discovered. While this assay is rather exquisitely designed to avoid perturbations to the specific signalling system being studied, by foregoing the need to stimulate cells with an agent such as forskolin, its specificity relies on PDE4 being the predominant PDE active in HEK293 cells. It is however, extremely effective when miniaturised, and very simple to employ once the cell lines have been created.

3.2.4 PDE Assaying Strategy

It is clear from the brief review of the available technology that the set up of an assay for PDE4 activity can be complex and time consuming. There is no perfect method, so the advantages and drawbacks of each approach must be contrasted, especially with respect to the time and funds available. It was decided that within the time restraints of a PhD project, previously well defined PDE assay systems that were easy to set up should to be used. This ruled out the creation, calibration and testing of a PDE activity sensing stable cell line, particularly as further genetic manipulations would be required to alter DISC1 expression status. This left two major options – to detect cAMP using a competition based commercial assay, and the lower throughput radiolabelled assay adapted from Marchmont and Houslay.

To perform a higher throughput screen to measure the effect of varying concentrations of a compound of interest on intracellular cAMP, the Perkin Elmer AlphaScreen was chosen, as there was an EnVision Multilabel plate reader available within the home Institute (Hupp group, Edinburgh Cancer Research Centre, CRUK). To provide a more accurate measure of factors specifically affecting PDE4 activity, the assay developed by Marchmont and Houslay in combination with 10μM rolipram was used, as this assay allows for assessment of PDE4 activity within an unaltered cellular context, and is relatively easy and quick to set up. The major drawback of this choice was that equipment in the radiation controlled area restricts the throughput of the experiment, allowing a maximum of 5 treated samples (in triplicate, plus and minus rolipram) and a negative control per experiment.

3.3 Results

3.3.1 The majority of PDE activity in SHSY5Y cells is PDE4 specific

Being initially derived from human neuroblastoma cells, it is presumed that SHSY5Y cells are a better model of neural environment than other cell lines available within the lab, for example HEK293 cells derived from human embryonic kidney. To ensure that SHSY5Y possess adequate endogenous PDE4 activity to assay, and discern the appropriate amount of protein to use in these assays, a simple standard curve of PDE activity was produced at increasing protein concentrations for SHSY5Y cells, using the PDE assay method described in 2.5.1 and 2.5.1.1. A comparative curve of endogenous PDE4 in HEK293 cells was also performed. The PDE4 specific component at each lysate concentration was discerned through the use of replicates containing 10μM rolipram as described previously.

In SHSY5Y cells the majority of the measurable activity was PDE4 specific. At 50μg of protein the assay was not yet saturated (Figure 3.2A). In HEK293 cell lysate, there was marginally less total PDE activity that in SHSY5Y (Figure 3.2B). Although there is marginally more PDE4 specific activity than non-PDE4 in HEK293, both amounts are sufficiently small that the error involved in measurements is much larger than in SHSY5Y cells. Furthermore, the assay did not saturate even at 75μg of protein.

An amount of 20μg of protein per sample was chosen for further work with SHSY5Y cells, as this is an easily obtainable amount of protein from small cell culture flasks and sits in the middle of the steepest section of the standard concentration curve. This maximises the potential for detecting changes in PDE4 activity in response to genetic or chemical manipulation of the cell line.

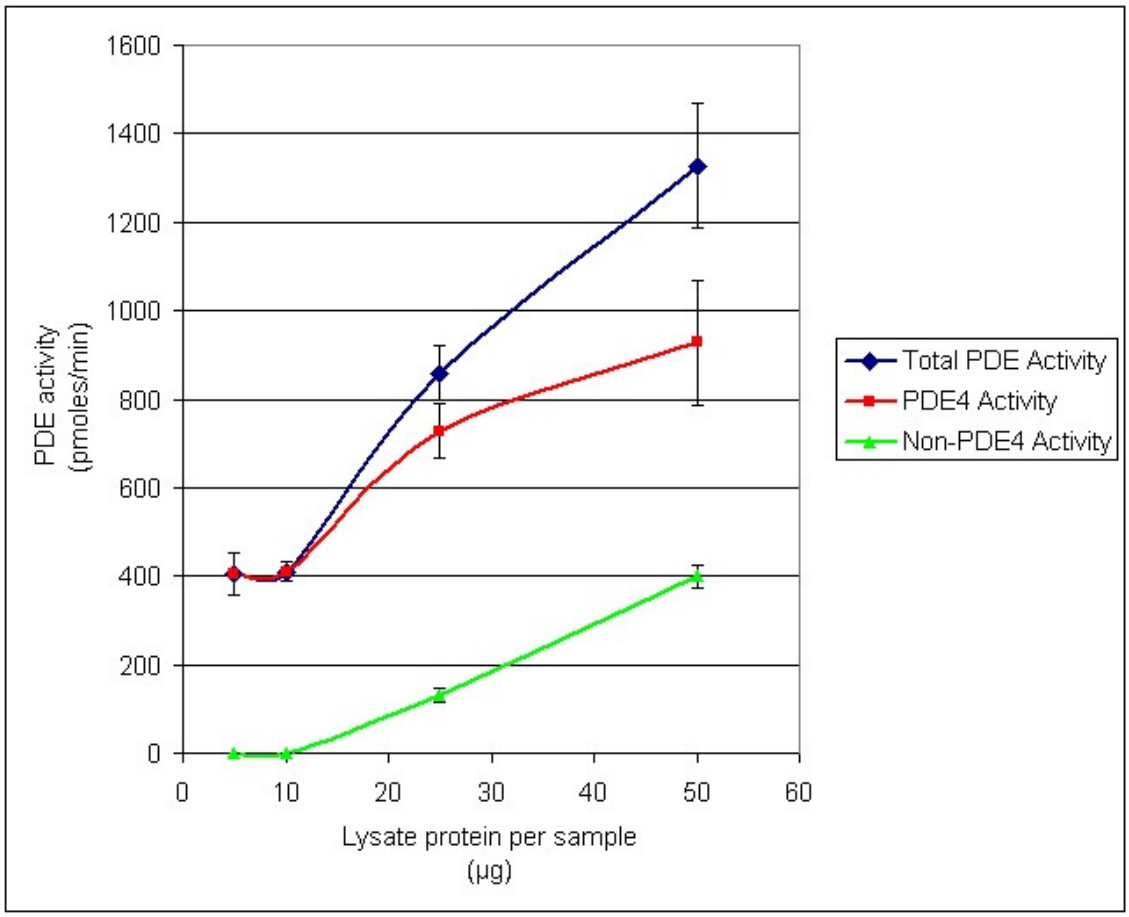


Figure 3.2A: A graph depicting the standard curve of PDE activity for increasing amounts of SHSY5Y lysate. PDE4 specific activity is inferred by the use of 10µM rolipram. SHSY5Y have a large range of measurable PDE4 activity, making them appropriate for detecting changes in PDE4 activity in response to genetic or chemical manipulations.

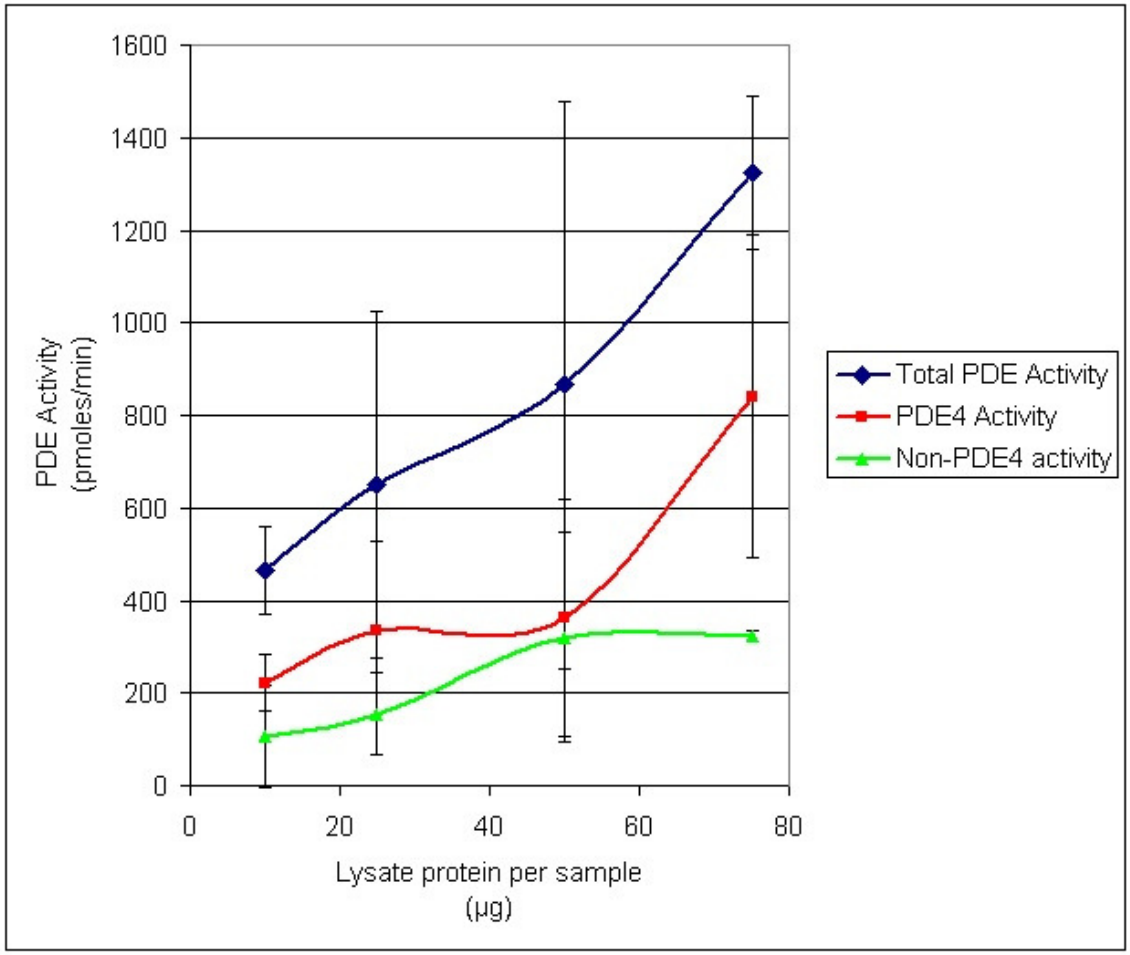


Figure 3.2B: A graph depicting the standard curve of PDE activity for increasing amounts of HEK293 lysate. PDE4 specific activity is inferred by the use of 10µM rolipram. HEK293 cells have slightly lower and more variable PDE activity than SHSY5Y cells, making them less useful to exploit with regards to PDE4 activity.

3.3.2 AlphaScreen Standard Curve

To assess that the EnVision Multi Label plate reader is correctly set up for AlphaScreen protocols, and assess the accuracy of the pipetting system being employed, a standard curve must be performed, as detailed in 2.5.2.1. This is a simple cell free experiment involving the preparation of a dilution series of cAMP, added to the donor and acceptor beads. A successful cAMP curve was obtained, using a manual CappAero 16 channel multi pipette, with very little error between triplicates (Figure 3.3).

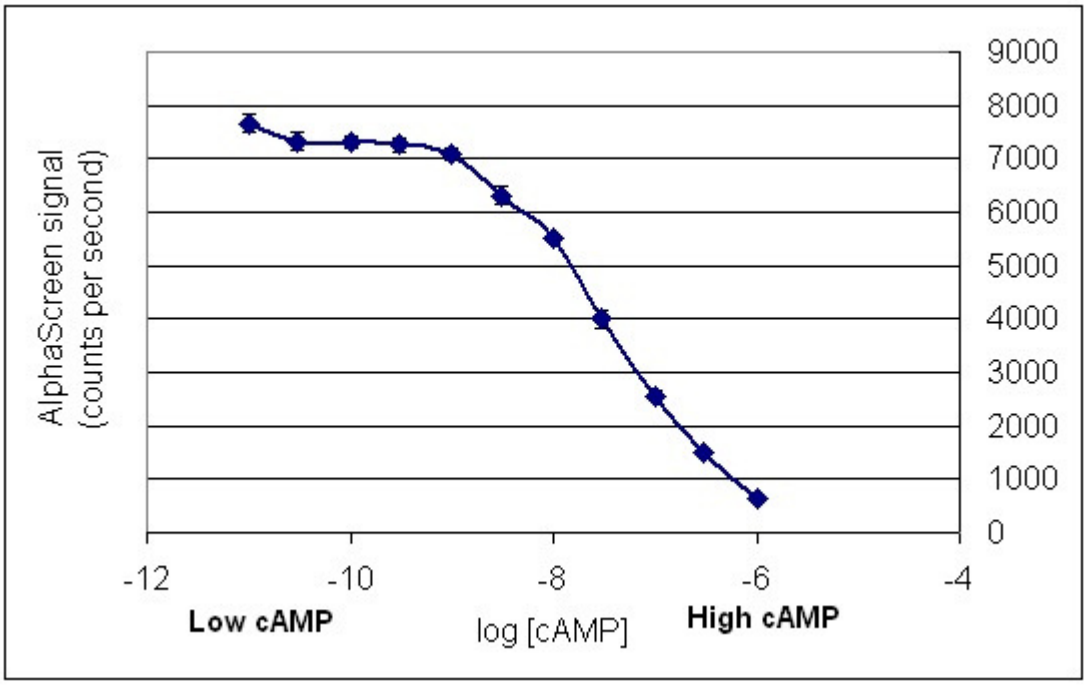


Figure 3.3: A standard cell free cAMP curve obtained from the AlphaScreen procedure on the EnVision MultiLabel Plate Reader. Error bars are shown, but are barely visible due to variation between triplicates being very small.

3.3.3 SHSY5Y density affects AlphaScreen efficacy

To define the number of cells to be used per well in the AlphaScreen assay, SHSY5Y cells were seeded at three different densities as described in 2.5.2.2. The standard AlphaScreen procedure using IBMX was performed as described in that section, where cells were stimulated with Forskolin to activate adenylate cyclase and increase cAMP production. After 30 minutes of treatment cells were lysed, and plates were read 4 hours later.

At a density of 10,000 cells per well, SHSY5Y cells produced a dose response to forskolin with a range of 10,000 AlphaScreen counts per second (Figure 3.4). A dose response was observed at lower densities with high concentrations of forskolin, but the

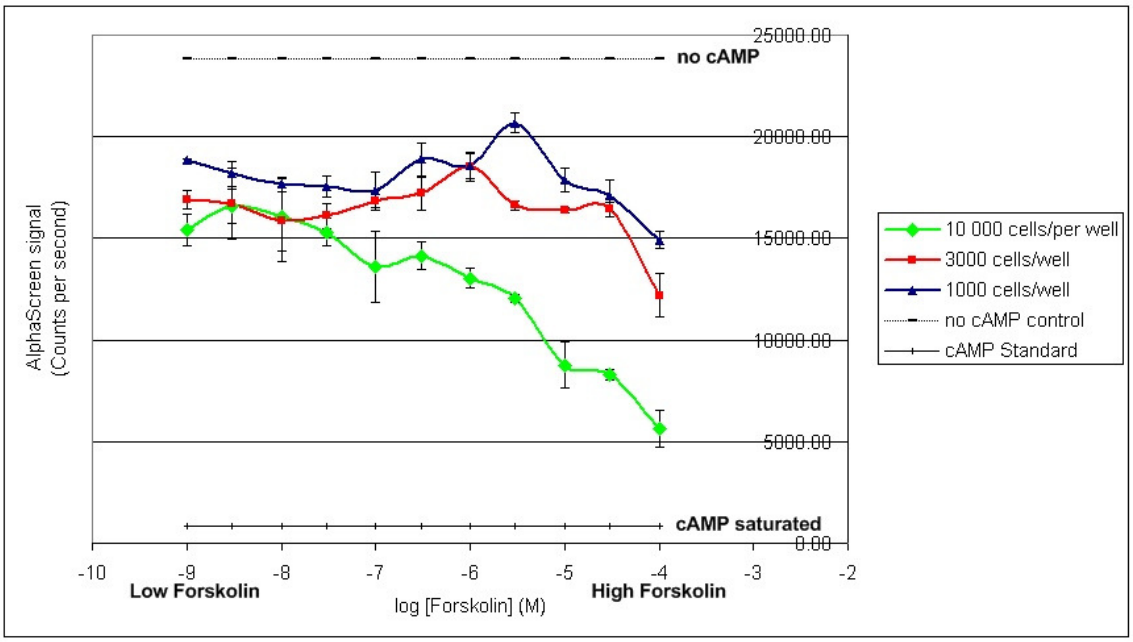


Figure 3.4: Graph showing the response of different density SHSY5Y cells to a dilution series of Forskolin. At 10,000 cells/well a good dose response with a large range of measurement is obtained. At lower densities, a response is only detectable at the highest concentrations of forskolin.

range was much smaller (~3000-5000 counts per second). This is most likely because the total cAMP in the low density wells was below the detection limit for the assay. For this reason, a cell density of 10,000 cells per well was chosen for further investigative assays.

3.3.4 Short term transfection of DISC1 variants

Increasingly in the research of genetics of psychiatric disease, attention has focussed on the presence of structural variants in DNA sequence, both common and rare, that may increase an individual's susceptibility to illness (Song et al., 2008). As these variants came to light relatively late in my project, it would have been useful to be able to transiently transfect variants of interest to assess their effect on PDE activity without the need for creation of a stable cell line. Transfection efficiency of SHSY5Y cells was investigated using the Nucleofector method described in 2.2.3.2, and a transfection rate

of 72% (+/-3.5) obtained over 4 experiments using the pmax optimised plasmid. Transfection rates with DISC1 GFP were significantly lower. Unfortunately, when PDE4 activity was measured, there was a large effect of transfection on PDE4 activity (37% decrease -/+3). As the assaying system used has a restricted throughput, it was not possible to perform a properly controlled experiment, and so this approach was discontinued. Only experiments using stable cell lines are featured in this thesis.

3.3.5 Creation of an inducible cell line overexpressing DISC1

Previous attempts in house to produce a stable cell line constitutively overexpressing DISC1 had proved unsuccessful. I was also unable to find evidence of other labs who had made successful attempts. For this reason the decision I produced an inducible stable cell line under the control of a tetracycline repressor, so that it was possible to "switch on" DISC1 expression 24 hours prior to experiments. The Invitrogen TRex system was used, which uses Tetracycline repressors to repress gene expression in the absence of tetracycline. Tetracycline binds to the tetracycline repressor homodimer and causes a conformational change that prevents the repressor from binding to the Tet operator controlling the expression of your gene of interest (http://tools.invitrogen.com/content/sfs/manuals/trexsystem_man.pdf).

Stable cell lines were created as described in 2.2.5, and overexpression of DISC1 tested initially by western blot with Zymed C-terminal antibody. Of the 10 clones tested, 2 showed strong expression of DISC1 after 24 hours treatment with Tetracycline (Figure 3.5) and had no leakage from the expression construct when tetracycline was absent. One of these clones was taken forward for further experiments (TRTODISC1). Overexpression of DISC1 mRNA was confirmed by Christoph Grünewald using qPCR on the iCycler (BioRad) to a level of 5.07 (+/-0.21, n=3) compared to uninduced cells (1.1 +/-0.28, n=3).

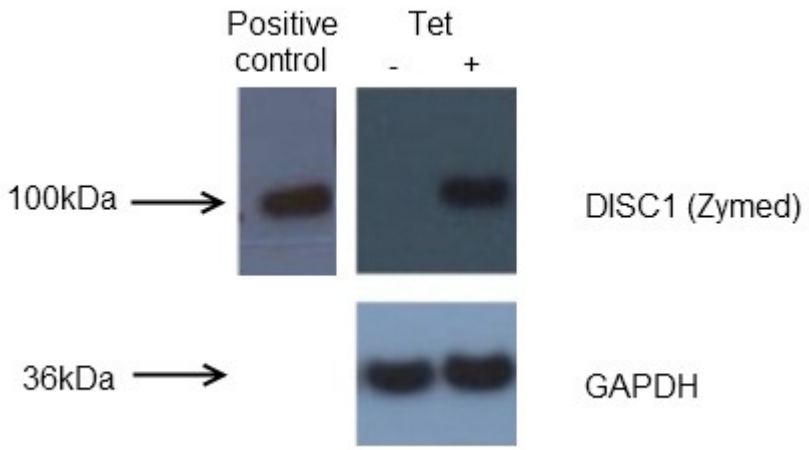


Figure 3.5: Western blot showing overexpression of the 100kDa form of DISC1 using the Zymed C-terminal antibody in TRTODISC1. The positive control shows transient transfection of the expression plasmid only into SHSY5Y cells. This clone has no leakage from the expression plasmid when tetracycline is not present.

3.3.6 DISC1, PDE4B and PDE4D localisation in stable cell lines

Immunostaining of endogenous PDE4B and DISC1 overlaps significantly in the cytoplasmic compartment of wild type SHSY5Y cells. To ascertain whether changes in DISC1 expression levels altered PDE4 localisation, immunocytochemistry was performed to label endogenous DISC1, PDE4B and PDE4D.

The αDISC1 antibody (kindly supplied by Professor Akiyama, University of Tokyo) was used to immunostain for DISC1, and displayed a heavily saturated signal when DISC1 was overexpressed, if the amplification gain of the laser used to image the uninduced cells was used. Overlap but not dependant colocalisation of DISC1 and PDE4B immunostaining was evident throughout the SHSY5Y cells regardless of DISC1 overexpression status (Figures 3.6A-F), and doesn't appear to be related to a single specific organelle. The signal from both proteins was increased in perinuclear regions. However, when the amplification gain was turned down to image the overexpressing

cells, it became evident that there was a large increase of nuclear signal from both DISC1 and PDE4B, when compared to the uninduced control cells (Figures 3.6G-I).

A very similar pattern of staining is observed with co staining of DISC1 with PDE4D. Again, there is an overlapping signal from both proteins throughout the cell, though low levels of staining within the nucleus (Figures 3.7A-F). The signal from both proteins is stronger in perinuclear regions, but PDE4D also shows strong staining in the cellular processes which contain neurofilaments and resemble primitive neurites (Glass et al., 2002). Overexpression of DISC1 again leads to an accumulation of both proteins in the nucleus, in contrast to the uninduced control (Figures 3.7G-I).

In the DISC1 knockdown line (created and provided by Fumiaki Ogawa, see section 2.2.6 for details), a very weak background signal is obtained from using the αDISC1 antibody. Again, overlap of DISC1 immunostaining can be seen throughout the control cells with both PDE4B and PDE4D, but is strongest in the perinuclear regions (Figures 3.8A-C, 3.9A-C). As would be expected, there is no observable colocalisation of signals when DISC1 is knocked down. Furthermore, the absence of DISC1 does not appear to grossly alter the cellular distribution of either PDE4B or PDE4D (Figures 3.8D-F, 3.9D-F).

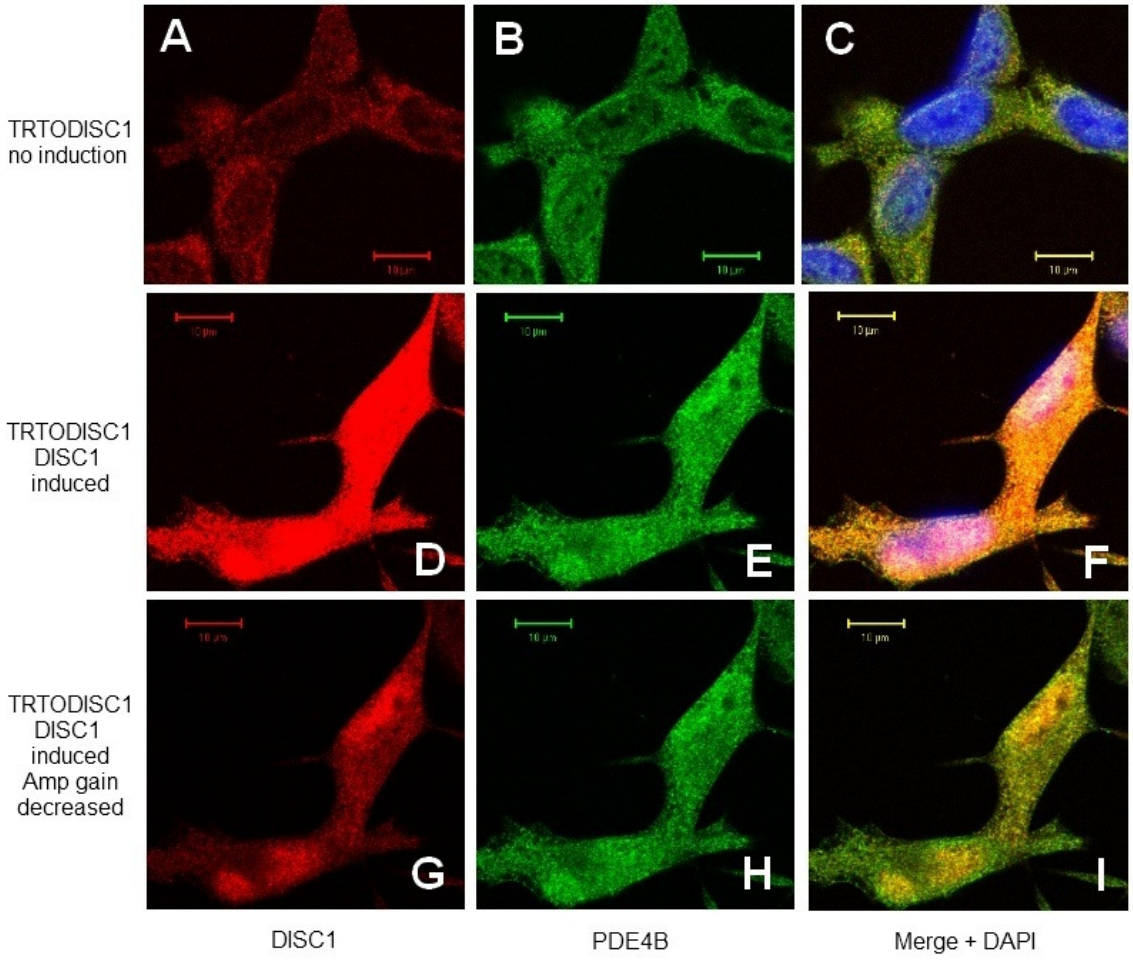


Figure 3.6 A-I: Immunocytochemistry of DISC1 and PDE4B in SHSY5Y cells. A-C shows the interaction in uninduced control TRTODISC cells. D-F shows the interaction in cells with 24 hours of DISC1 induction, with the amplification gain of the laser maintained at the same level as for the uninduced cells. In G-I, this gain is turned down to reveal an accumulation of DISC1 and PDE4B in the cell nucleus.

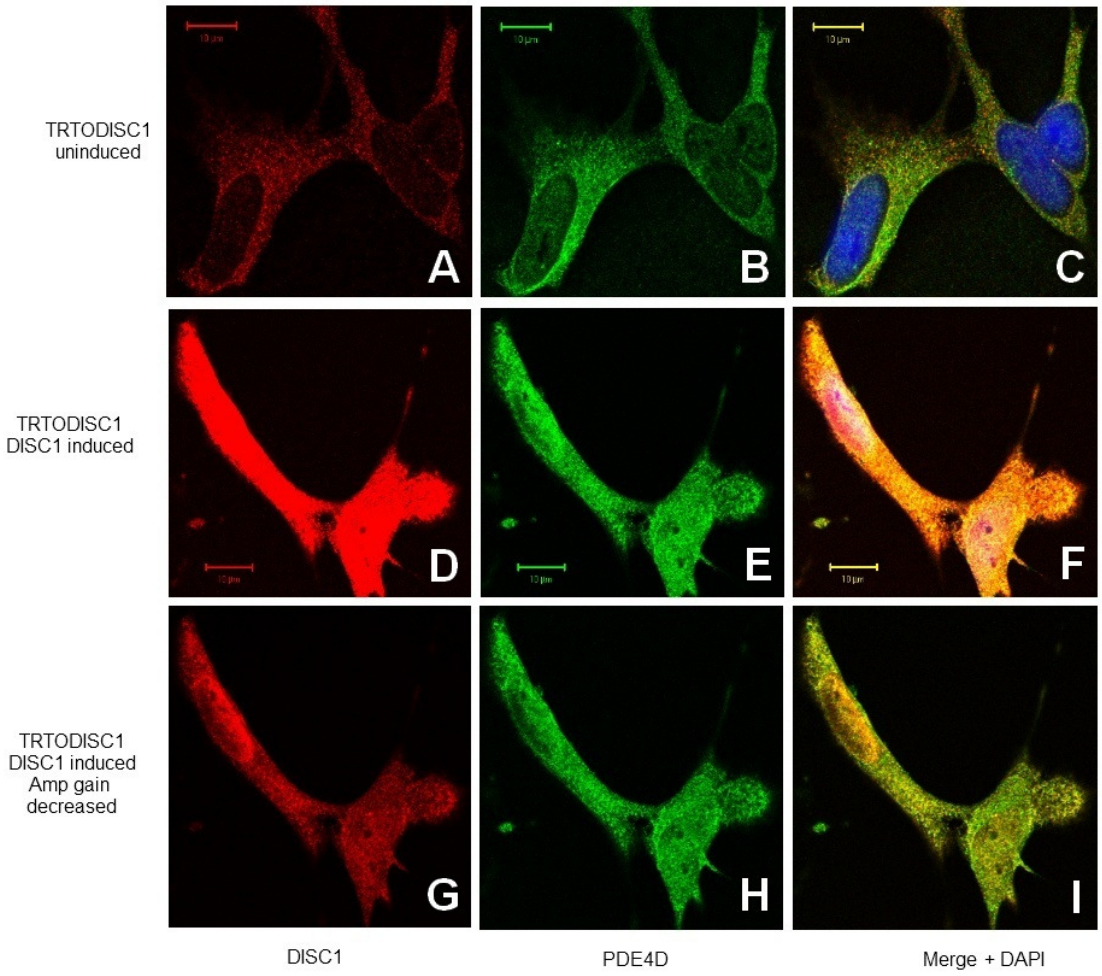


Figure 3.7 A-I: Immunocytochemistry of DISC1 and PDE4D in SHSY5Y cells. A-C shows the interaction in uninduced control TRTODISC cells. D-F shows the interaction in cells with 24 hours of DISC1 induction, with the amplification gain of the laser maintained at the same level as for the uninduced cells. As with Figure 3.6, in G-I, this gain is turned down to reveal an accumulation of DISC1 and PDE4D in the cell nucleus.

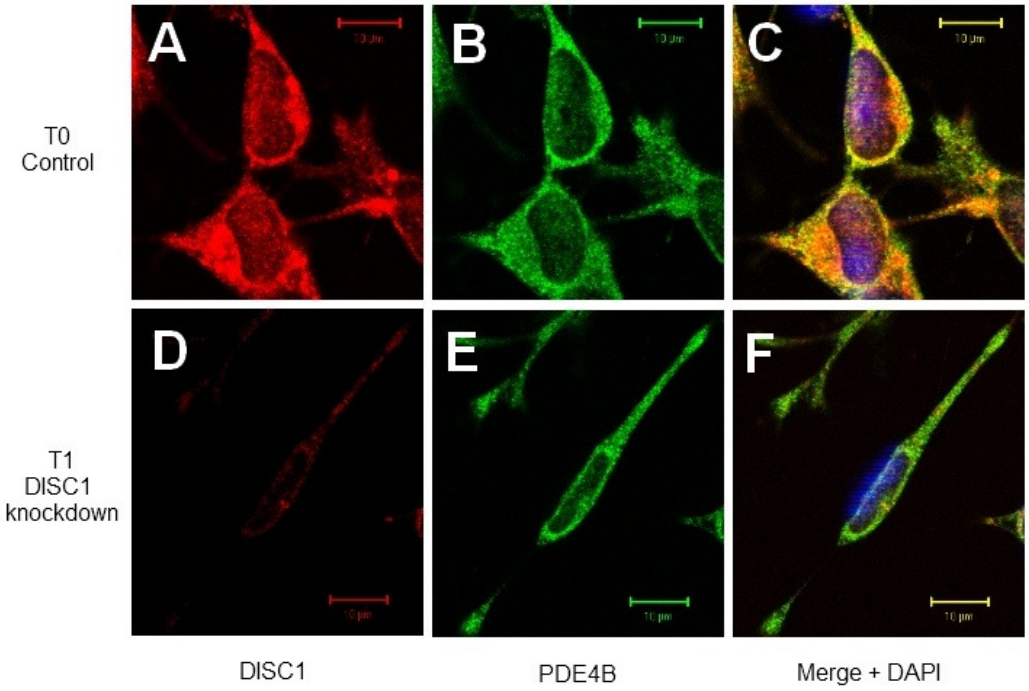


Figure 3.8 A-F: Immunocytochemistry of DISC1 and PDE4B in T0 (A-C), the control scramble stable cell line, and T1, the DISC1 knock down cell line (D-F).

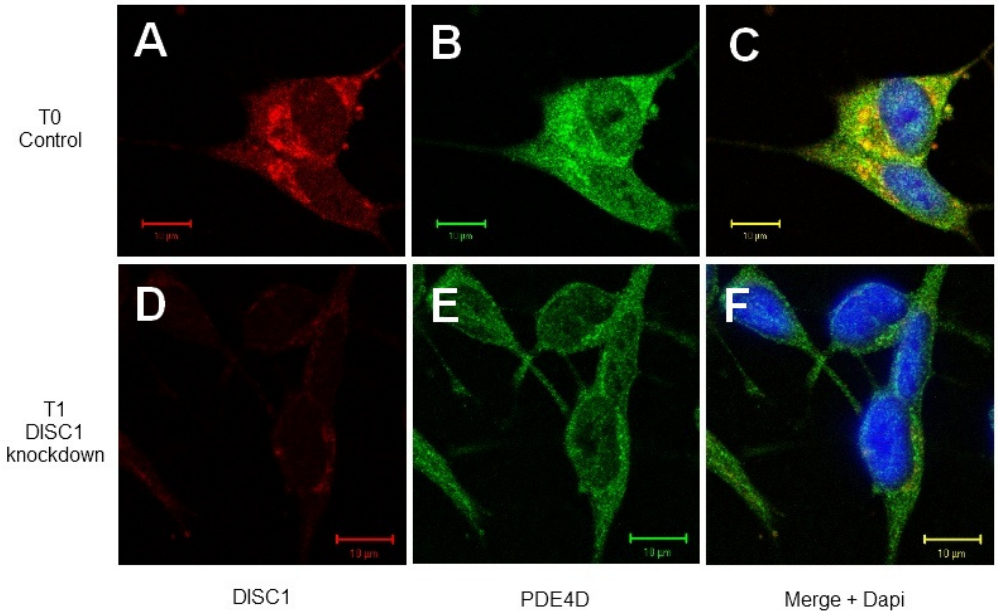


Figure 3.9 A-F: Immunocytochemistry of DISC1 and PDE4D in T0 (A-C), the control scramble stable cell line, and T1, the DISC1 knock down cell line (D-F).

3.3.7 The expression of PDE4 mRNA in stable cell lines

The major issue with the use of stable cell lines is that long term adaptive changes may take place in these cells that could effect the expression of related genes. It was important to eliminate changes in PDE4 isoform expression as being causal in any measured change in PDE4 activity. This was likely to be more of an issue in the DISC1 knockdown cell line supplied by Fumiaki Ogawa, as this was a constitutive knockdown of DISC1. However, gene expression changes would also be possible after 24 hours of DISC1 induction in the overexpressing cell line, so it was prudent to check in both cell lines. 3 sets of matched cDNA (T0 scramble vs T1 knock down, and TRTODISC1 with and without tetracycline) were kindly provided by Christoph Grünewald.

3.3.8 Primer design for quantitative RT PCR by the iCycler

Primers were designed using the NCBI Primer Blast Tool as described in section 2.4.1. Human PDE4A-D subfamily sequences were aligned using ClustalW, and a pan primer (Pan PDE4A, Pan PDE4B etc) for each gene identified by defining a point downstream of the isoform specific N termini. NCBI Primer Blast tested and suggested suitable primer pairs where at least one primer crosses an exon exon junction, and generates a PCR product in cDNA of 150-200 base pairs. Primers were ordered and tested on the iCycler using the method described in section 2.4.3. Melt curves were obtained from PDE4A, 4B and 4D, no melt curve was obtained from PDE4C. The PCR products were run on a gel to ensure a product of the correct size had been formed, and it was confirmed that there was no PCR product in the PDE4C reaction (Figure 3.10). Basic PCR was performed on human heart and brain cDNA (kindly provided by Christoph Grünewald) with the PDE4C primers pairs, under the same cycle conditions as qPCR. A PCR product of the correct size was obtained from heart and brain cDNA, but not SHSY5Y (Figure 3.11). This suggests that the PDE4C isoform is not present at levels detectable by qPCR in SHSY5Y cells.

Once this initial test had been performed, standard curves were obtained for each of the successful primer pairs as described in 2.4.4. Successful standard curves were obtained

for each of the primer pairs, although PDE4A and PDE4B had efficiencies at the top of the acceptable range (Figure 3.12).

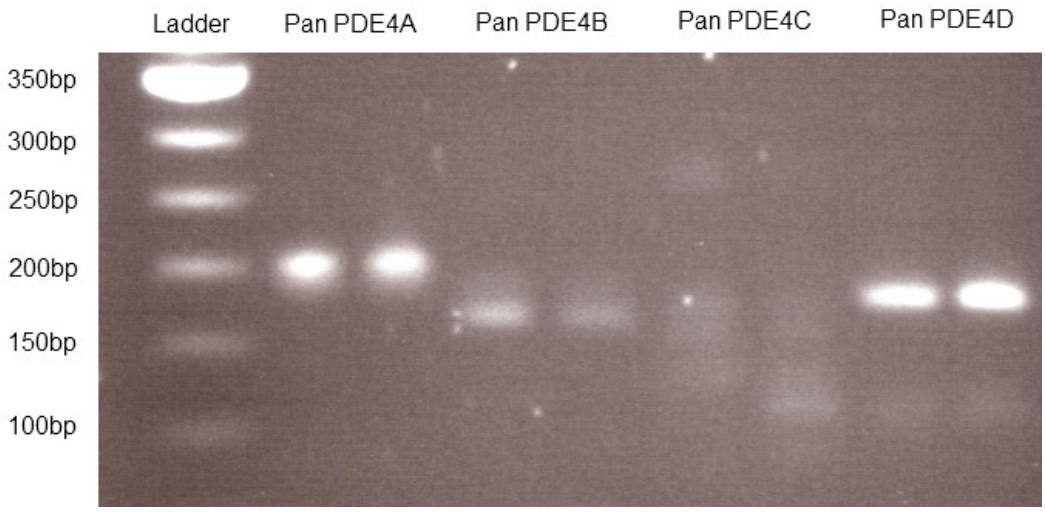


Figure 3.10: Diagram showing the gel on which the pan PDE4A, B, C and D primer products were run, to confirm correct product size. PDE4A should have a product size of 188bp, PDE4B 150bp, PDE4C 161bp and PDE4D 171bp. All primer pairs produced products of the correct size, except for PDE4C where no product was detected on the iCycler or gel.

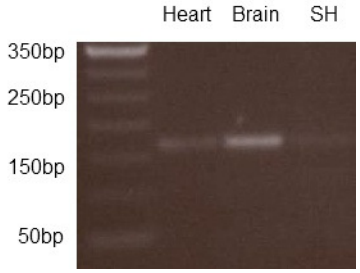
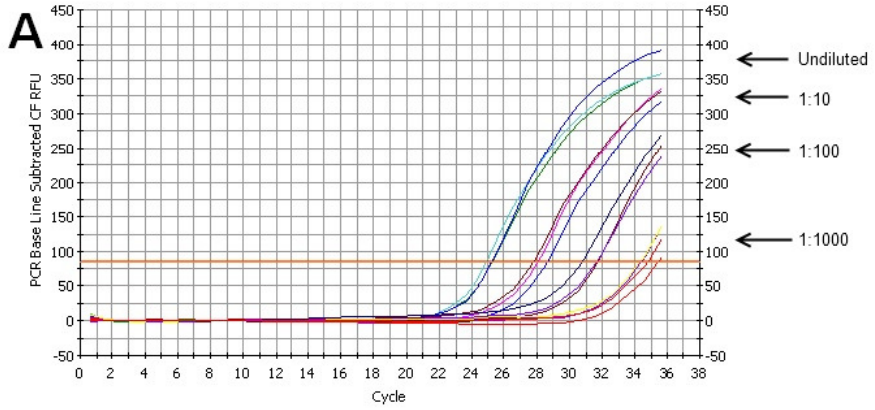
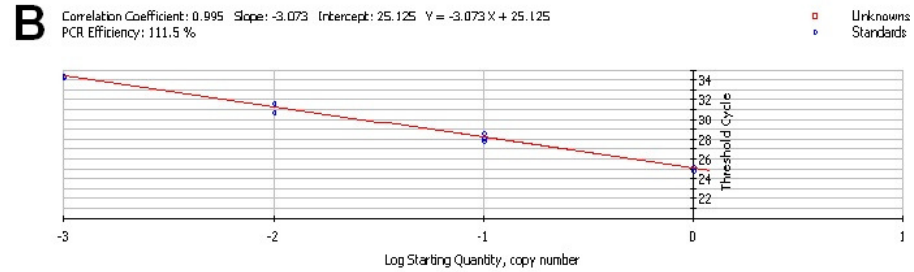
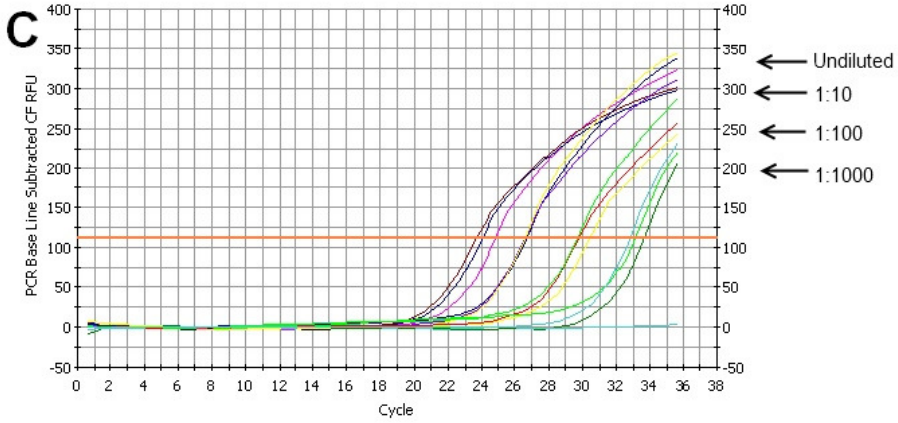


Figure 3.11: A PCR product of the expected size of 161bp is obtained when basic PCR is performed on human heart and brain cDNA using PDE4C primers. No product is obtained from using the same primer pairs on SHSY5Y cDNA.


A
PCR Base Line Subtracted CF RFU
Cycle
Undiluted
1:10
1:100
1:1000


B
Correlation Coefficient: 0.995 Slope: -3.073 Intercept: 25.125 Y = -3.073 X + 25.125
PCR Efficiency: 111.5 %
Unknowns
Standards
Threshold Cycle
Log Starting Quantity, copy number


C
PCR Base Line Subtracted CF RFU
Cycle
Undiluted
1:10
1:100
1:1000


D
Correlation Coefficient: 0.993 Slope: -3.042 Intercept: 23.920 Y = -3.042 X + 23.920
PCR Efficiency: 113.2 %
Unknowns
Standards
Threshold Cycle
Log Starting Quantity, copy number

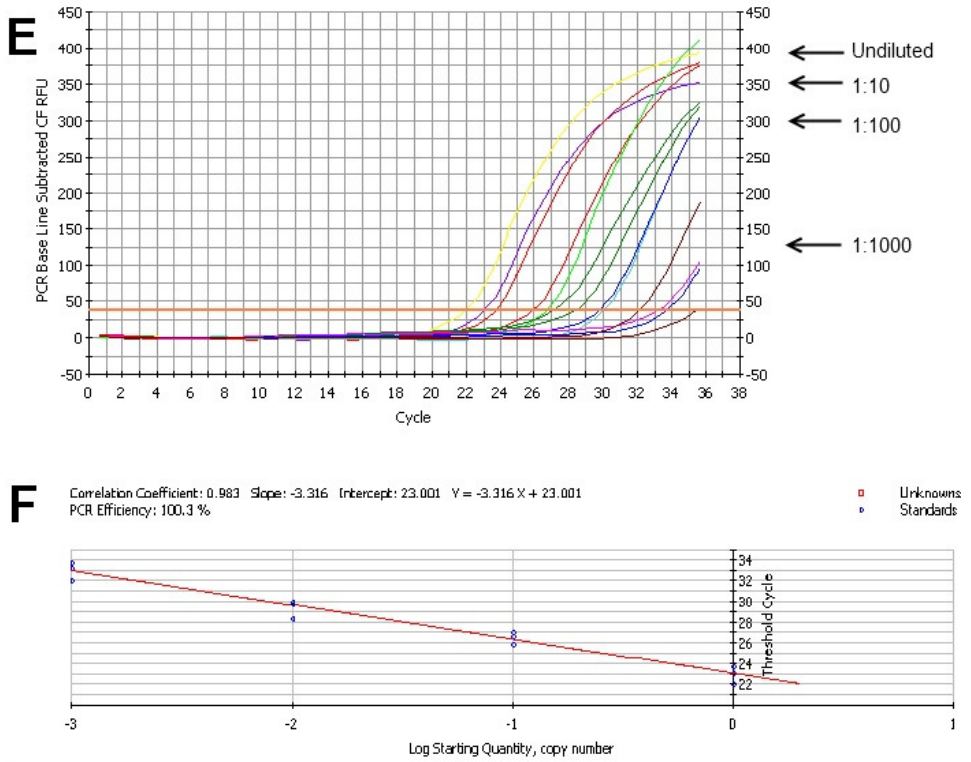
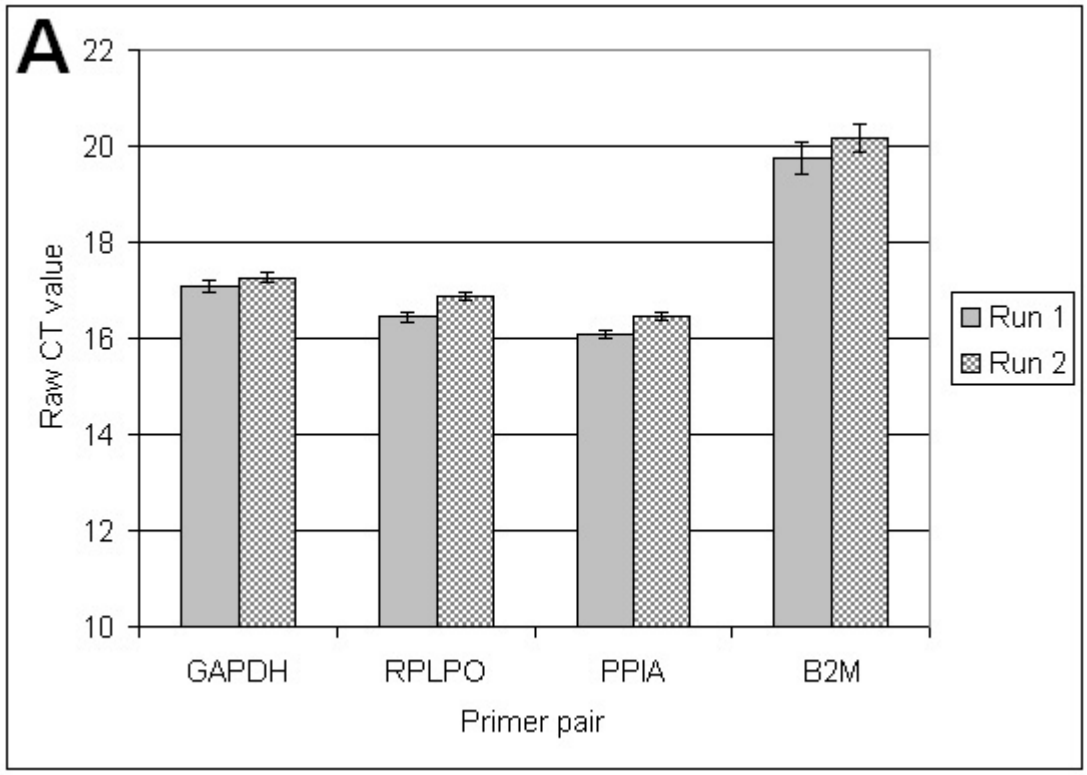


Figure 3.12 A-F: Standard cDNA dilution curves for PDE4A (A and B), PDE4B (C and D) and PDE4D (E and F) primer pairs. In all three cases, the upper graph depicts the amplification of product per PCR cycle. The lower graph is the standard curve graph, depicting logged cDNA dilution against CT values (the cycle number where product was detected above the threshold marked on the upper graph), and thus the efficiency of the primer pair.

3.3.9 qPCR Data Analysis

The relative expression of PDE4A, 4B and 4D in TROTODISC1 with and without 24 hours of induced DISC1, and in T0 and T1, the DISC1 knockdown line, was assessed in 3 separate paired cDNA samples. The entire experiment was performed twice, resulting in two technical replicates of each sample. Raw CT values were obtained for each of the three primer pairs, alongside values from each cDNA sample for 4 reference genes: GAPDH, RPLPO, PPIA and B2M. Primer pairs for these genes were previously tested and supplied by Christoph Grünewald. Raw CT values for the reference genes were input into NormFinder (Andersen et al., 2004), to assess the stability of reference genes

throughout the experiment. In both replicate experiments, B2M proved highly unstable, and so was excluded from further analysis (Figure 3.13).



B

Primer pair	Stability Value
GAPDH	0.16
RPLPO	0.35
PPIA	0.28
B2M	0.95

Figure 3.13A: Graph depicting the raw CT values for each reference gene across both RTPCR experiments. The greater variability in B2M can be clearly seen by the increased size of the error bars. Table 3.13B depicts the stability values of each reference gene as found using NormFinder. A stability value of 0 indicates no variation between samples, and increasing values correspond to increasing variation. B2M has a much higher value than the other three primer pairs, and was removed from further analysis.

After excluding B2M, the lowest raw CT value for each primer pair was identified and the relative expressions were converted to quantities (W_R) using a modification of the $2^{-\Delta\Delta C}T$ method:

$$W_R=(1+E)^{(CT^{min}-CT^{x})}$$

where W_R is the quantity for relative expression for one sample, E is the previously determined primer pair efficiency, CT^{min} the previously determined lowest raw CT value, and CT^{x} the raw CT value of the well of interest. If the primer pair was 100% efficient, (E=1) then the PCR product would double with every qPCR cycle. This formula allows for a correction to be performed when the efficiency is not 100%.

The next step in the analysis involves normalising these relative expression values to the geometric mean of the three remaining housekeeping genes (GAPDH, RPLPO, PPIA) in each sample. Finally, a fold change was obtained for each matched sample pair by division (T1/T0 and TOTRDISC1 plus Tetracycline / TRTODISC1 no Tetracycline). The arithmetic mean of the 3 fold changes was obtained for each experimental run. To combine the two technical replicates, the arithmetic mean of the two experiments was obtained, and the overall standard error calculated:

$$SEM=[(SEM_1)^2 + (SEM_2)]^{1/2}/2$$

Where SEM equals the total standard error of the mean for the experiment, SEM_1 the SEM from the first run and SEM_2 the standard error for run 2.

3.3.10 The expression of PDE4 isoforms in SHSY5Y based stable cell lines

There was no difference in expression of PDE4A, PDE4B or PDE4D with regards to the DISC1 status of the SHSY5Y based cell lines (Figure 3.14). There is considered to be no biologically meaningful difference in expression when the fold change value lies between 0.8 and 1.2, and all means fell within this range. There was, however, more variation between samples in the constitutive knock down line than in the inducible cell line after 24 hours of DISC1 induction.

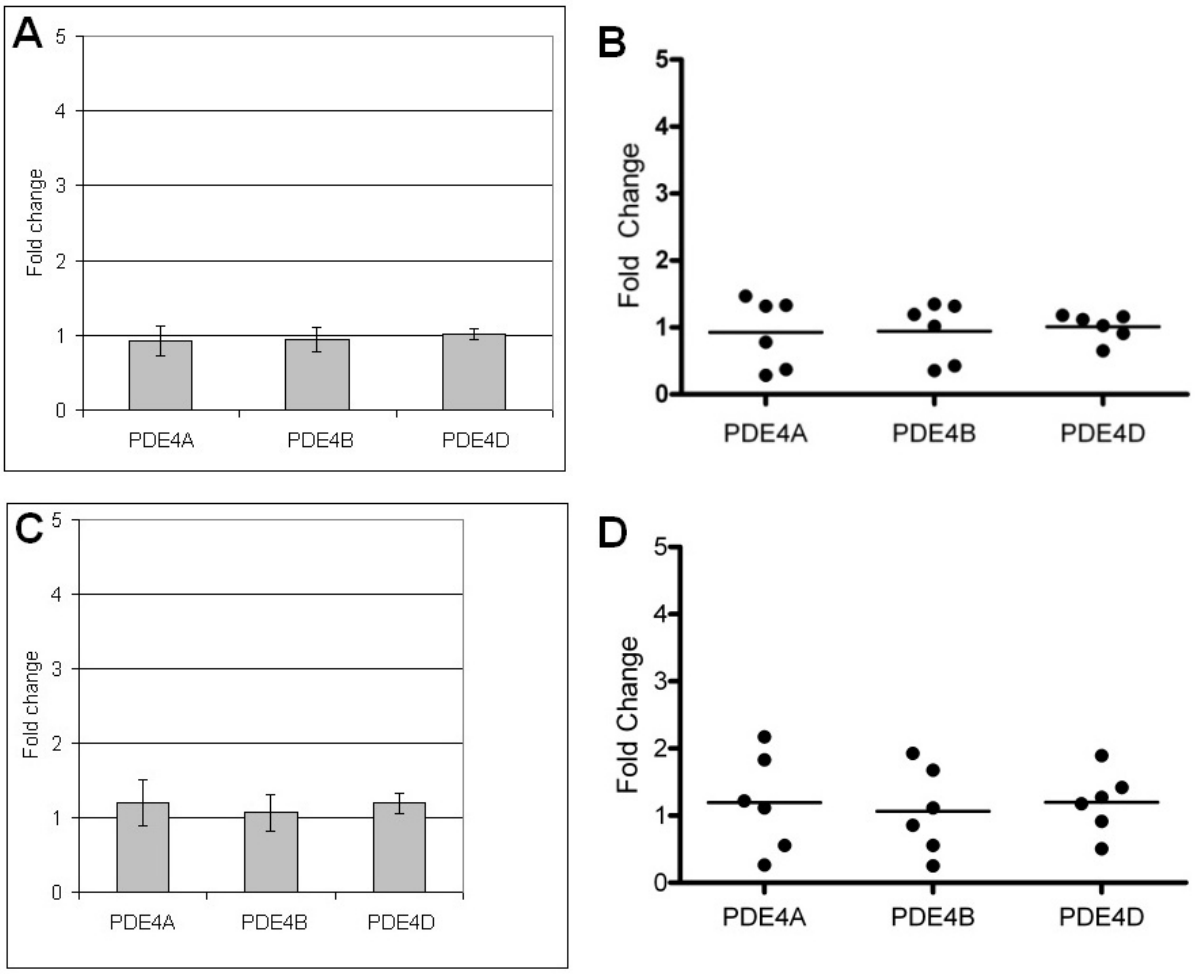


Figure 3.14 A-D: A shows the fold changes of PDE4 isoforms in TRTODISC1 with induction of DISC1 in comparison to uninduced cells. B shows the same data as a scatter plot of individual matched samples. C shows the fold changes of PDE4 isoforms in the T1 knockdown line compared to the T0 scramble control line. Again, D shows the same data as a scatter plot of fold changes from individual matched samples. All mean values lie within the range of fold changes generally accepted as “no change:” 0.8-1.2.

3.4 Discussion

As yet, there is no ideal means for detecting changes to PDE activity in the endogenous context of a cell. Whilst means to measure compounds and cellular processes that have effects on PDE4 activity have improved quite significantly over recent years, there remains no direct way of measuring activity *in situ*. Recent advances in genetic reporter systems and high throughput cAMP accumulation assays allow for measurement of PDE4 activity *in situ*, but with perturbed expression of important pathway components, or indirect measurement through cAMP accumulation respectively. For this reason a dual approach was chosen, using resources already available in house, of medium throughput AlphaScreen cAMP accumulation assays combined with low throughput PDE activity assays as described earlier.

SHSY5Y cells were validated as an appropriate model system for investigating changes in PDE4 activity. They are a well established, well characterised cell line derived from a human neuroblastoma and used regularly in cell culture experiments as a model for signalling in neurons (some examples include Smith et al., 2006, Olivieri et al., 2003, Smart et al., 1995) as they are easier to prepare and less time consuming to grow than primary neuron cultures. SHSY5Y are also less differentiated than primary neurons, which makes them a somewhat simpler system to use in early exploratory experiments, reducing the complexity resulting from having neurons with specific functional regions (axons, dendrites, spines, cell bodies). SHSY5Y are known to endogenously express PDE4 isoforms (Jang IS, 2001), and addition of the PDE4 specific inhibitor Rolipram to cultures results in a large scale increase in intracellular cAMP (Morgan et al., 1993), suggesting PDE4 is constitutively active. Endogenous 71kDa DISC1 interacts dynamically with PDE4B1 in SHSY5Y cells (Millar et al., 2005b). Similar interactions were later observed with exogenous DISC1 and PDE4D3 (Murdoch et al., 2007). I have demonstrated that the majority of measurable PDE activity in these cells is PDE4 specific, and that they are very responsive to forskolin challenge, providing an excellent workable range with which to study changes in PDE4 activity. The increases due to

forskolin are at the lower range of published fold changes, and thus tentative assumptions can be made as to the composition of the active PDE4s in these cells. It appears that there may be a combination of short form PDEs, which are unresponsive to PKA phoshorylation as they lack the appropriate consensus site, and the responsive long form PDE4s.

The main drawback of this cell type is that they are relatively difficult to transfect by standard chemical methods, but this can be surmounted to some extent by the use of nucleofection. However, exploratory experiments using this type of transient transfection procedure showed a significant effect of transfection on PDE4 activity, thus this approach was deemed inappropriate for further investigation. The remainder of the results throughout the thesis are performed on stable lines with inducible DISC1 or constitutive DISC1 knockdown.

The localisation of PDE4B, PDE4D and DISC1 was assessed to investigate whether a change in the DISC1 expression status of the stable cell lines would lead to a change in localisation of these proteins. In all cells expressing DISC1 substantial overlap of DISC1 and PDE4B immunostaining was seen throughout the cell, with the strongest signal arising from perinuclear regions, as seen previously (Millar et al., 2005b). While knockdown of DISC1 resulted in no gross changes to PDE4B and PDE4D localisation, overexpression of DISC1 resulted in an accumulation of DISC1 and both PDE4s in the nucleus, where little signal was seen previously. It is unclear from previous work what the cause of this movement may be: full length DISC1 possesses a clear nuclear localisation signal, and a nuclear enriched 75-85kDa form of DISC1 has been reported (Sawamura et al., 2005), but there is no reason to suspect that expression of this form of DISC1 would be favoured in these cells. Indeed, western blots of over expressing cells show a dominant signal at the predicted 100kDa (see Figure 5.2 for an example).

Characterisation of the expression of PDE4 mRNA was performed by qPCR on the iCycler using pan isoform probes. As expected from studies on brain tissue (Engels et

al., 1995, Iona et al., 1998), the pan PDE4C probe failed to produce a PCR product even at high cycle numbers, suggesting there is little or no PDE4C expressed in SHSY5Y cells. Expression of PDE4A, PDE4B and PDE4D was clearly detectable. qPCR showed that, consistent with previous in house microarray work on the DISC1 translocation family lymphoblastoid cell lines and DISC1 mutant Q31L and L100P mice (Camargo *et al*, paper in preparation, Brown *et al*, paper in preparation), changes in the DISC1 status of SHSY5Y cells (24 hours of DISC1 overexpression and constitutive knockdown) had no effect on the expression of PDE4 mRNA. Any differences observed in these cell lines are thus likely to be directly due to changes of activity in PDE4s, and not an indirect measure of a change in PDE4 expression. This makes these cell lines as excellent model for studying changes to PDE4 activity with regards to DISC1, and common neuroactive chemical compounds.

4 The effect of Short Term Forskolin and Lithium treatment on endogenous PDE4 activity

4.1 Introduction

It is well accepted that the treatment of cells with forskolin activates adenylate cyclase, increasing production of cAMP and activating PKA signalling (Seamon et al., 1981, Scott, 1991). Exogenously transfected long PDE4 isoforms are activated by PKA phosphorylation following treatment with forskolin in COS7 cells. PDE activity increases 1.4-1.6 fold from baseline on forskolin treatment, using the same approach as used in this thesis (MacKenzie et al., 2002). The first part of this chapter describes an experiment to establish the range of endogenous PDE4 response to a 10μM forskolin challenge in SHSY5Y cells. This concentration of forskolin produced a robust increase in cAMP concentration in this same cell line using the AlphaScreen protocol (Figure 3.4).

The main body of this chapter follows on from recent work which demonstrated the binding and inhibitory activity of DISC1 on GSK3β (Mao et al., 2009). Using an anti DISC1 antibody, GSK3β was immunoprecipated from E14 foetal mouse brain lysate. GST pulldown assays reported two regions of DISC1, amino acids 1-220 and 356-595, that bound to GSK3β. Of these two fragments, 1-220 demonstrated the ability to inhibit autophosphorylation of GSK3β *in vitro* in a dose-dependent fashion. Surface Plasmon resonance was used to further define this binding site to within residues 211-225 (Mao et al., 2009). This area overlaps the first common binding site of PDE4B and D on DISC1, identified by peptide array, which lies between amino acids 192-239 (Murdoch et al., 2007). The interesting possibility is therefore raised of competitive binding or co regulation of these two enzymes, through their interaction with DISC1.

GSK3β is an enzyme intricately involved with AKT signalling, via inhibitory phosphorylation by AKT of Ser9 on GSK3β (Cross et al., 1995). This association has been independently implicated in the pathogenesis of schizophrenia by observations that AKT1 protein levels and inhibitory phosphorylation of GSK3β are both decreased in the frontal cortex and hippocampus in schizophrenic cases (Emamian et al., 2004a). GSK3β is also strongly implicated in psychiatric illness by virtue of its inhibition by LiCl, a common mood stabiliser (Beaulieu and Caron, 2008) and primary treatment for Bipolar Disorder. It is still unclear whether there are single or multiple molecular mechanisms by which lithium exerts its effects. At present there are two major pathways implicated: disruption of inositol homeostasis and the aforementioned inhibition of GSK3β.

As discussed in the introduction, GSK3β was originally proposed as a target of lithium after observations that the teratogenic effects of lithium on Xenopus embryos were markedly similar to the effects resulting from disruption or inhibition of GSK3β and wingless (Wnt) signalling. Lithium has an IC_{50} of 2mM for inhibition of bacterially purified GSK3β *in vitro*. In *in vitro* enzyme reaction studies however, LiCl became more potent at inhibiting GSK3β as the Mg^{2+} concentration decreased, implying that the IC_{50} of the enzyme may vary depending on the magnesium content of the tissue (Ryves and Harwood, 2001).

Since these initial experiments, pharmacological and genetic manipulation of GSK3β related pathways has shown behavioural effects relevant to lithium treatment. GSK3β heterozygous knock out mice recapitulate the behavioural effects of lithium treated wild types, demonstrating both decreased immobile time in the forced swim test and decreased "hole pokes," in an exploratory behaviour paradigm (O'Brien et al., 2004). Brain specific expression of a constitutively active form of GSK3β results in abnormal behaviour of transgenic mice, including decreased food intake, increased locomotor activity, hyper-responsiveness to acoustic startle and decreased immobility in the forced swim test. Overexpression of GSK3β disrupts regulation of various components of the

AKT signalling pathway (Prickaerts et al., 2006). DISC1 also binds and alters the function of KIAA1212 (Kim et al., 2009b, Enomoto et al., 2009), which directly binds AKT to increase activation by phosphorylation at Thr^{308} and Ser^{473} of AKT (Anai et al., 2005). AKT in turn is an upstream regulator of GSK3β (Cross et al., 1995), suggesting the possibility for multiple regulatory cell signalling circuits of GSK3β and a role for DISC1.

Finally, there is also evidence that cAMP signalling acts upstream of GSK3β. Studies in zebrafish highlight the importance of prostaglandin E2 activity for increasing β-catenin stability, leading to the formation of larger pools of haematopoetic precursor cells. Treatment of zebrafish with indomethacin, a non selective cycloxygenase inhibitor, results in a decrease in numbers of haematopoetic stem cells, which is rescued by treatment with forskolin. This rescue effect is removed by adding H89, a PKA inhibitor, suggesting a role for cAMP/PKA signalling upstream of prostaglandin mediated Wnt signalling. In lethally irradiated mice (a process which ablates bone marrow), GSK3β inhibition increases the rate of bone marrow repopulation, whereas indomethacin treatment has the opposite effect. In *ex vivo* whole bone marrow, PGE2 activation and forskolin treatment results in both raised cAMP and increased stability of β-catenin. A concomitant change (the direction of this changed is not stated, but an increase is assumed) is observed in phosphorylation of Ser9 of GSK3β in these cells, but this experiment was unable to establish whether this is a direct effect of cAMP fluctuations (Goessling et al., 2009). Forskolin increases phosphorylation of Ser9 of GSK3β, decreasing GSK3β activity by 50% and preventing apoptotic death due to KCl withdrawal in primary cerebellar granule cells. These effects are blocked by administration of H89 to cultures, but are independent of ERK and AKT signalling. Indeed, purified PKA directly phosphorylates purified GSK3β *in vitro*, although immunoprecipation experiments *in vivo* have not proved a direct interaction (Li et al., 2000).

There are multiple reasons therefore, to hypothesise that PDE4 activity may alter as part

of a series of regulatory loop responding changes in the activity of cAMP/PKA, AKT and GSK3β. The finding that DISC1 interacts and inhibits the activity of GSK3β (Mao et al., 2009), a protein linked to multiple processes involved with psychiatric disease including synaptic plasticity, cell survival, CREB and Wnt signalling (reviewed Beaulieu and Caron, 2008), is tantalising. To investigate the hypothesis that GSK3β activity may affect PDE4 activity, I performed a series of assays measuring cAMP accumulation and enzyme activity in the presence of LiCl and SB216763 (a GSK3β specific inhibitor).

4.2 Results

4.2.1 The effect of forskolin on endogenous PDE4 activity in SHSY5Y cells

Wild type SHSY5Y cells were treated for 30 minutes with 10μM forskolin or vehicle, lysed and assayed for PDE4 activity as described in 2.3.2 and 2.5.1. This treatment led to a highly significant 1.4 fold increase in PDE4 activity (Figure 4.1, +/-0.05, p=0.02, n=3). This magnitude of effect is comparable to the previously reported activity level of endogenous PDE4B1 transfected into COS7 cells, despite use of a lower concentration of forskolin (MacKenzie et al., 2002). This suggests that at 10μM forskolin, maximal PKA mediated PDE4 activation is occurring.

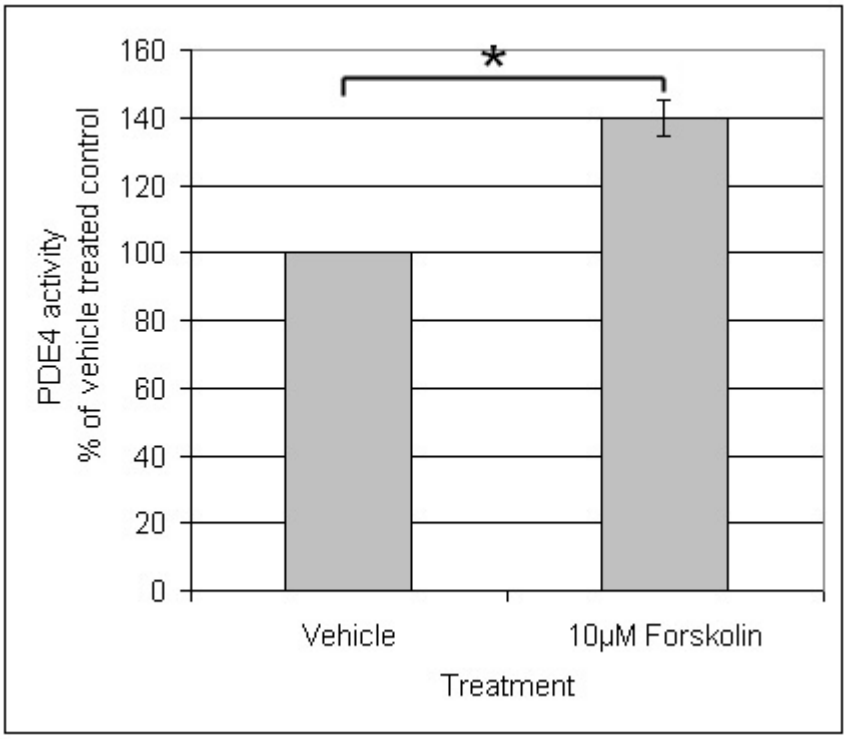


Figure 4.1: The effect of treatment with 10μM forskolin on endogenous PDE4 activity in SHSY5Y cells. A highly significant increase is observed. *p=0.002, n=3. Mean PDE4 activity in the vehicle treated control is 108.4 pmoles/mg/min.

4.2.2 The effect of Lithium on cAMP accumulation in SHSY5Y cells

SHSY5Y cells were AlphaScreened for cAMP accumulation in response to 30 minutes treatment with varying concentrations of LiCl (from 0-10mM), without or without the presence of Forskolin as described in 2.5.2.3. LiCl treatment at concentrations above 5mM resulted in an increase in cAMP levels (Figure 4.2A), which was highly significant at 10mM (p=0.00002, n=4, summarised in Figure 4.2B). There is a non significant trend in the inhibition observed at 3mM in this experiment. In this experiment the inhibitory effect of lithium was masked by treatment with 10μM forskolin, which stimulates adenylyl cyclase to increase production of cAMP at the cell membrane, activating PKA signalling. Results are displayed as relative to control as there was not sufficient cAMP standard available to produce a full standard curve with each assay.

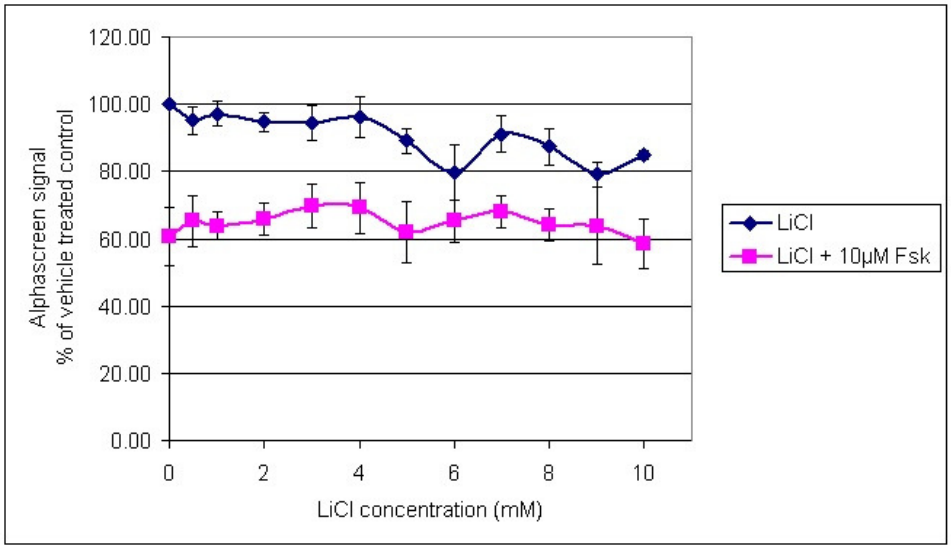


Figure 4.2A. Graph showing the effect of LiCl treatment on cAMP accumulation in SHSY5Y cells. cAMP concentration is plotted as relative to the signal obtained by vehicle treated SHSY5Y cells.

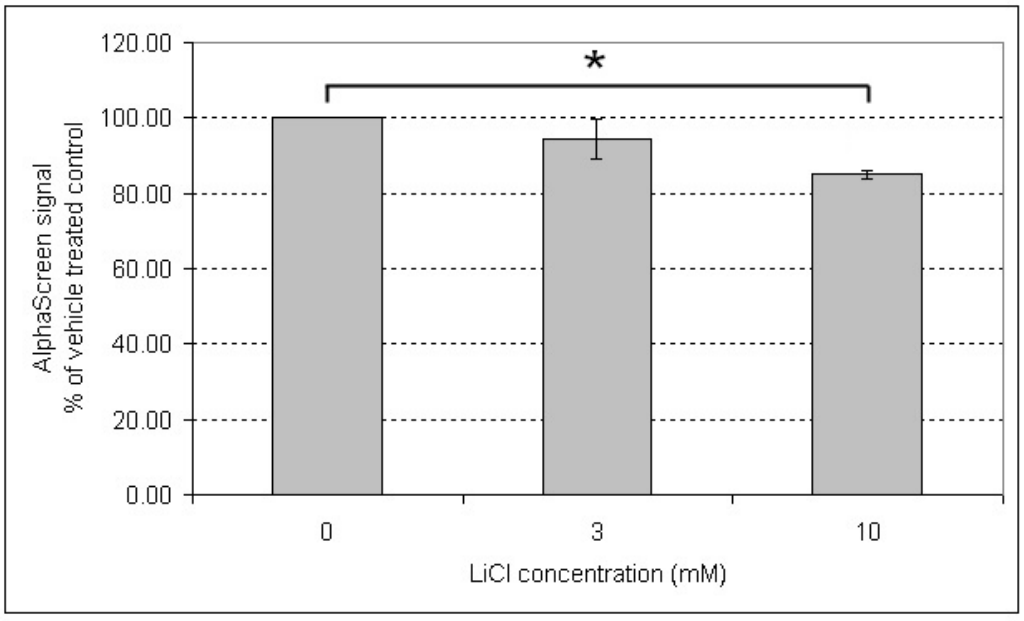


Figure 4.2B: Graph to summarise the effect illustrated in figure 4.2A of short term LiCl treatment on cAMP levels in SHSY5Y cells, as measured by AlphaScreen. *n=4, p=0.00002

This finding is open to several interpretations. The two most obvious to consider are that LiCl treatment acts on a pathway that affects the rate at which adenylyl cyclase produces cAMP. The second is that it acts to inhibit the activity of phosphodiesterase enzymes, which break down cAMP. Early work on the effect of lithium treatment on adenylyl cyclase on treated membranes and brain slices suggested that lithium actually inhibits the production of cAMP through inhibition of adenylyl cyclase (Ebstein et al., 1980, Newman and Belmaker, 1987). It later became apparent that only three of the ten isoforms of adenylyl cyclase were actually susceptible to this inhibition. Isoforms II, V and VII are all susceptible to inhibition by low concentrations of LiCl (1-2mM), but the magnitude of this effect varies depending on the presence of Forskolin or concomitantly overexpressed dopamine type 1 receptors (Mann et al., 2008). While little work appears to have been done on assessing the isoforms of adenylyl cyclase present in SHSY5Y cells, pharmacological characterisation of M_3 muscarinic receptor stimulation of the aforementioned cells suggests the presence of isoform I, and perhaps VIII (Hirst and Lambert, 1995), which would mean they were not susceptible to this effect on adenylyl cyclase. There is no evidence in the literature that LiCl would lead to an increase in AC activity, as is potentially being seen here.

The other possibility is that LiCl treatment at this concentration is, either directly or indirectly, altering the activity of the phosphodiesterase enzymes which break down cAMP. Inhibition of these enzymes would lead to an increase in cAMP levels. For this reason I performed a simple experiment to assess this possibility.

4.2.3 The effect of Short Term Lithium Chloride treatment on Phosphodiesterase 4 activity

Low throughput PDE assays were used (Marchmont and Houslay, 1980) to directly assess the effect of LiCl treatment on PDE activity. Serum starved wild type SHSY5Y

cells were treated for 30 minutes with either vehicle or LiCl at 3mM and 10mM concentrations, and assayed as described in 2.5.1.

Short term treatment of SHSY5Y cells with 3 and 10mM concentrations of LiCl results in a decrease in PDE4 specific activity in a range of magnitude very similar to the changes seen in cAMP levels (Figures 4.2B and 4.3A). This decrease in activity is highly significant at the 10mM concentration (p=.005, n=3). There is no trend observed in the non-PDE4 activity in the same lysates (Figure 4.3B), which comprises a small fraction of the measurable PDE activity (Figure 3.2A). It is therefore likely that inhibition of PDE4 activity is responsible for the increase in cAMP observed in the AlphaScreen.

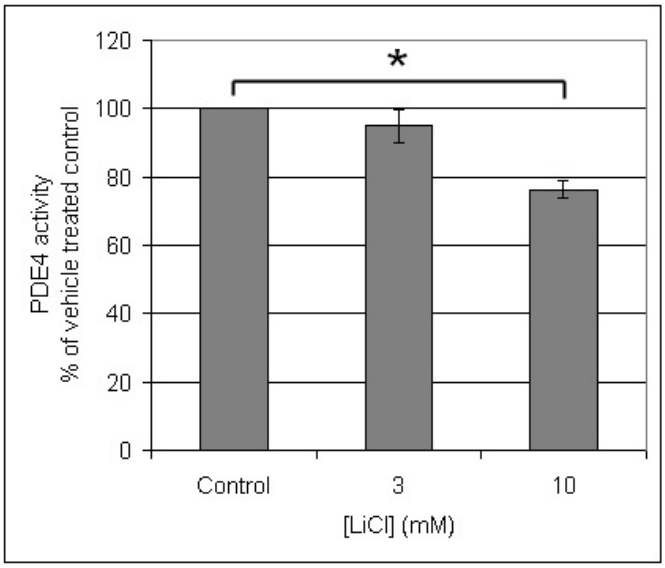


Figure 4.3A The effect of short term LiCl treatment on PDE4 activity in SHSY5Y cells. Treatment with 3mM LiCl leads to a non significant decrease in PDE4 activity, while treatment with 10mM LiCl results in a highly significant decrease in activity. * N=3, p=0.005 Mean PDE4 activity of the vehicle treated control is 104.9pmoles/mg/min.

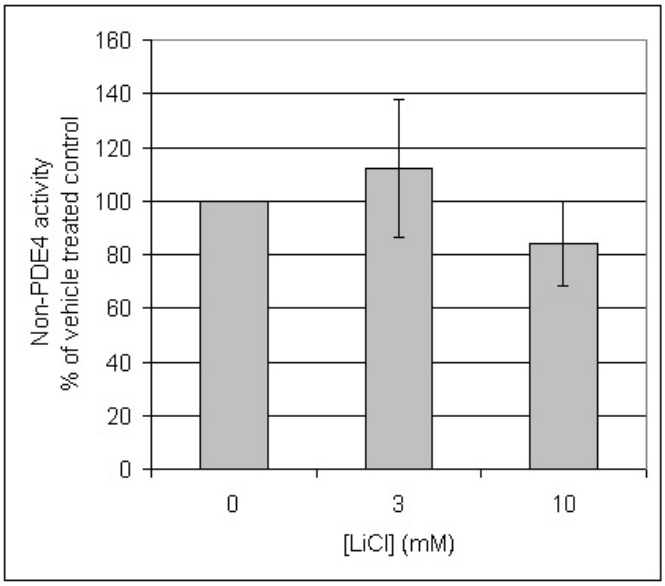


Figure 4.3B - The effect of short term LiCl treatment on non-PDE4 activity in SHSY5Y cells. There are no significant effects of LiCl treatment. Mean non-PDE4 activity in the vehicle treated control is 26.3 pmoles/mg/min.

As mentioned previously, LiCl is a drug with many known targets (Beaulieu and Caron, 2008), and potentially some others which are unknown. There is no evidence in the literature that Lithium directly inhibits PDE4, but PDE4 does have a magnesium co-factor (Saldou et al., 1998), and hence there is hypothetical potential for this to occur (Hallcher and Sherman, 1980, Ryves and Harwood, 2001). Alternatively, and perhaps more likely, it may be that PDE4s are controlled through the actions of GSK3β, one of the major known targets of LiCl. GSK3β has multiple downstream effectors, and thus it is highly possible that through one of these pathways, an unknown pathway, or the interaction with DISC1, that GSK3β also regulates PDE4 activity.

4.2.4 The effect of GSK3β specific inhibitors on PDE4 activity

Low throughput phosphodiesterase assays were performed to compare the inhibition of PDE4 by 10mM LiCl with 3μM SB216763, a compound that inhibits GSK3β with a Ki

of 9nM by competing with ATP (Coghlan et al., 2000) with minimal effects at other similarly structured cyclin dependent kinases (CDKs).

Serum starved SHSY5Y cells were treated with either vehicle (DMSO at a final concentration of less than 0.1%), 3μM SB216763, 10mM LiCl or a combination of LiCl and SB216763. Cells were incubated for 30 minutes, before assaying as described earlier. Cells treated with 3μM SB216763 showed a significant decrease ($p=0.003$, $n=3$) in PDE4 activity, equal in magnitude to treatment with 10mM LiCl (Figure 4.4A). There was a small, but non-significant additive effect of combining both compounds, suggesting that GSK3β was responsible for most, if not all of the inhibition observed. Conversely, as with LiCl treatment, SB216763 had no effect on non-PDE4 activity (Figure 4.4B). Combining both compounds produced a non significant decrease in non-PDE4 activity, similar to the decrease due to LiCl observed earlier in Figure 4.3B.

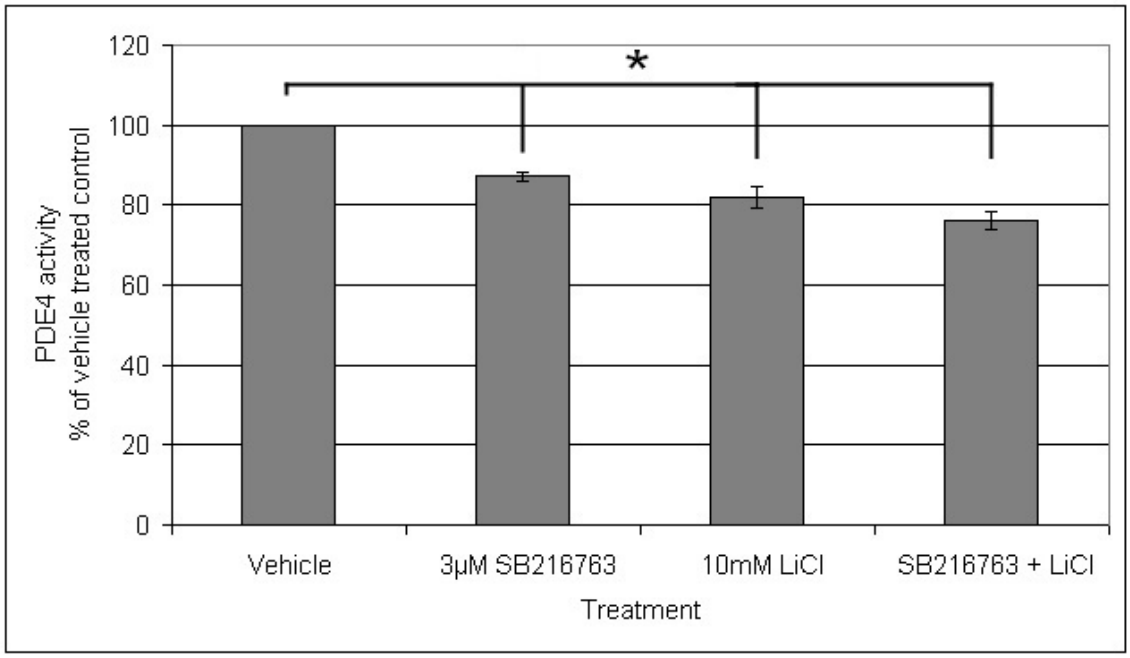


Figure 4.4A: The effect of the GSK3β specific inhibitor SB216763 and 10mM LiCl on PDE4 activity. *n=3, $p<0.01$ in all cases. Mean PDE4 activity in the vehicle treated control is 130.2 pmoles/mg/min.

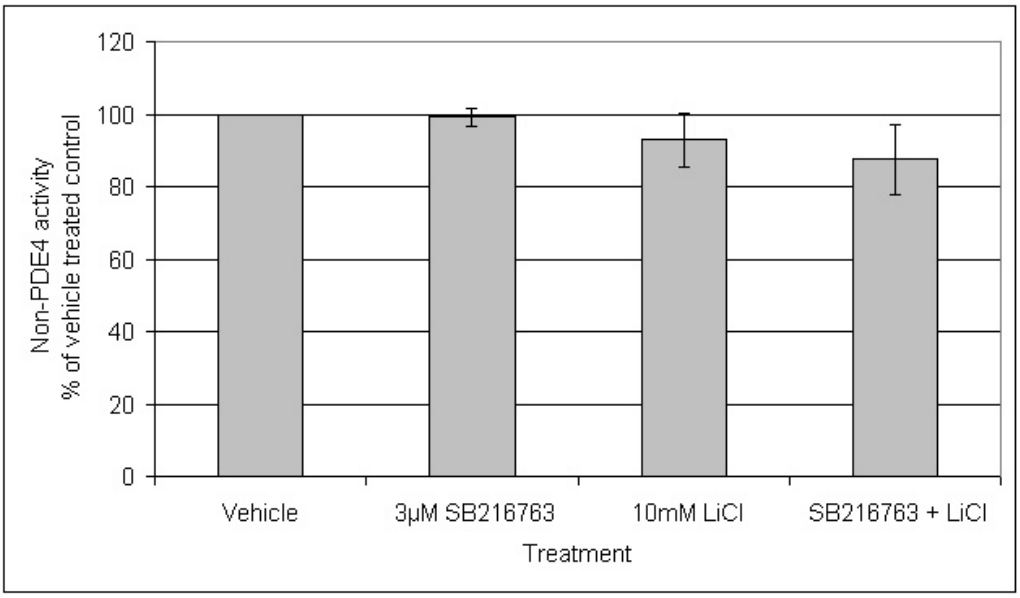


Figure 4.4B: The effect of 3μM SB216763 and 10mM LiCl on non-PDE4 activity, as dissected out by addition of 10uM Rolipram. None of the changes depicted are approaching statistical significance after 3 experiments. Mean non-PDE4 activity in the vehicle treated control is 27.5pmoles/mg/min.

4.2.5 Colocalisation of exogenous tagged GSK3β and PDE4B in nucleofected SHSY5Y cells

Due to problems finding an anti GSK3β antibody suitable for endogenous immunocytochemistry, the experiment was performed using cotransfected myc tagged GSK3β and flag tagged PDE4B1. pcDNA3.1-myc-GSK3β and pcDNA3.1-Flag-PDE4B1 were generated and kindly supplied by Fumiaki Ogawa. SHSY5Y cells were cotransfected with the expression constructs by nucleofection as described in 2.2.3.2 and seeded onto baked glass coverslips at a density of $5x10^5$ cells per well. These coverslips were incubated overnight, then fixed and immunostained as described in 2.3.5. As a control, untransfected cells were also labeled with the primary antibodies and secondary antibodies alone. Neither of these controls showed significant non-specific staining (data not shown).

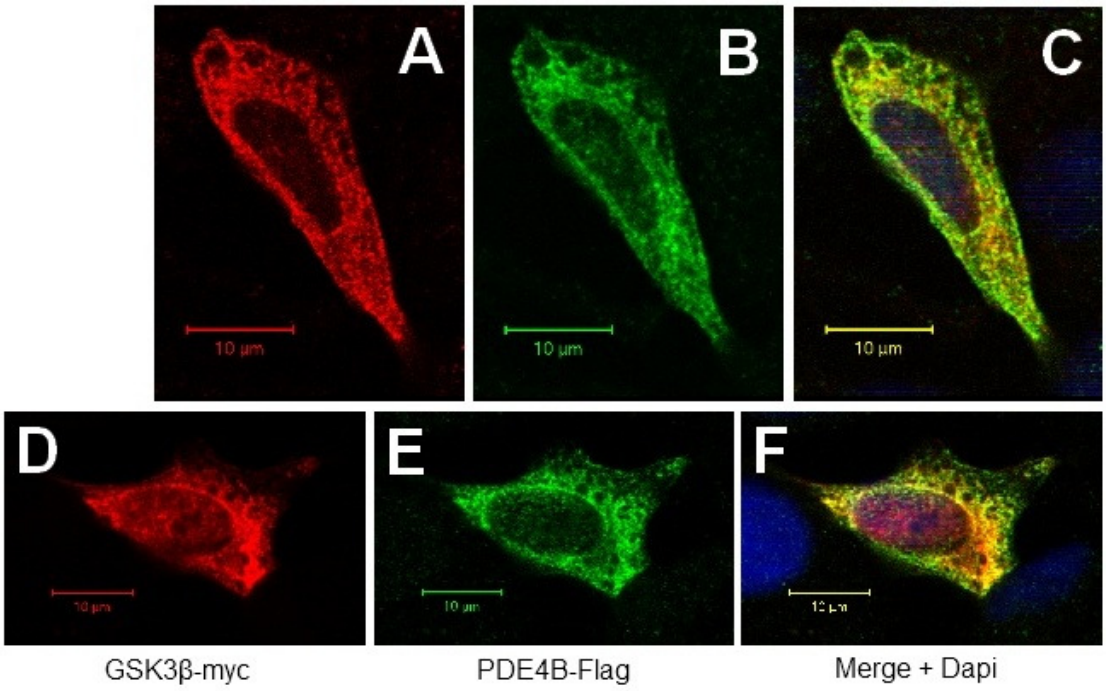


Figure 4.5: Immunocytochemistry showing the localisation of myc tagged GSK3β (A and D) and flag tagged PDE4B1 (B and E) contransfected into wild type SHSY5Y cells.

Colocalisation was clearly evident between these two exogenously expressed proteins (Figure 4.5). As observed previously with endogenous PDE4B, PDE4D and DISC1, both signals were strongest in perinuclear regions, with minimal signal from nuclear regions. The mitochondria, endoplasmic reticulum and microtubules all cluster in a similar distribution within this region, but staining in this experiment is unlikely to relate to a single one of these organelles as they all have very specific pattern of distribution not evident here (http://www.cellsignal.com/catalog/organelle.html). Endogenous DISC1 and PDE4B have been shown previously in this region to colocalise with mitochondrial staining in a small subset of SHSY5Y cells (Millar et al., 2005b).

Cellular fractionation in SHSY5Y cells and neurons has demonstrated the presence of GSK3β in purified mitochondria as well as in cytoplasmic fractions (Bijur and Jope, 2003, Hoshi et al., 1996), thus it is clearly possible that colocalisation of PDE4B and GSK3β is occurring, amongst other organelles, in the mitochondria.

4.2.6 Do PDE4B and PDE4D contain canonical GSK3β phosphorylation sites?

Sequences of human PDE4B and PDE4D isoforms were aligned using ClustalW, and run through a series of phosphorylation prediction databases as detailed in Materials and Methods. No highly scoring potential canonical GSK3β phosphorylation sites were discovered in these databases (Figures 4.6A-D). However, it is becoming increasingly apparent with GSK3β that the presence of other proteins in a complex may significantly alter the phosphorylation capacity of the enzyme (Ikeda et al., 1998). While this means it is unwise to rule out PDE4B as a direct target for GSK3β, it also means that further studies to investigate this possibility are made more complex. Complicating matters yet further, GSK3β often requires phosphorylation of a priming proximal serine or threonine 4 residues away from the residue of interest, making direct studies mixing recombinant forms of the protein difficult (Fiol et al., 1987, Jope and Roh, 2006, Thomas G, 1999). Whether the effect observed of GSK3β phosphorylation is direct or via the involvement of other proteins is therefore still unclear, and outside the scope of this investigation.

In the PDE4B isoforms Scansite, the only database searched that distinguishes between GSK3α and GSK3β phosphorylation sites, detected potential GSK3β sites in PDE4B3, but these were only detected by the lowest stringency scan. These sites clustered at residues 42, 46, 50 and 54, with an outlier at residue 87, in an arrangement providing the potential for priming that can favour phosphorylation by GSK3β. Scansite noted GSK3β specific sites, again only on a low stringency search, at residues 55 and 59 of PDE4D5 and PDE4D3. These residues are found on the same background sequence in both of these isoforms, but ClustalW has aligned them differently. Again, these residues are the correct distance to allow for priming.

A series of other potential sites were detected by the other phosphorylation databases, all of which were GSK3 specific, except for Disphos 1.3. Disphos 1.3 was used because this takes the disorder of a protein into an account to assess the accessibility of a putative phosphorylation site. In PDE4B1, a three site cluster was detected at residues 145, 150 and 154, and these sites are fully conserved in PDE4B3, with partial conservation near the N terminal of B2. The other sites detected in more than one database have no potential priming site, and all have low likelihood scores (Figure 4.6C).

In the PDE4Ds there is a cluster of potential sites found between the PKA phosphorylation site and the common DISC1 binding site, at conserved residues corresponding to 202, 207 and 211 in PDE4D4, which would allow for priming phosphorylation. As one can see from Figure 4.6B, there are two other 2 residue clusters, but again these have noncanonical spacing for a priming site. Once more, all sites have low likelihood scores (Figure 4.6D).

I compared these results to those observed in a substrate of GSK3β with well defined phosphorylation sites: β-catenin. In this protein, priming phosphorylation is required by a different kinase at Ser45, before GSK3β phosphorylates residues Thr41, Ser37 and Ser33 (Hagen and Vidal-Puig, 2002). This is a similar clustering to that described above in PDE4B1, PDE4B3 and PDE4D4. The human β-catenin protein sequence was obtained from NCBI protein database (Ref Seq NP_001895.1) and run through the 5 phosphoprediction tools used for the PDE4 isoforms. It is likely that this sequence was used to train DisPhos1.3 and KinasePhos2.0, as Ser33 and Ser37 received scores of 1 in both databases, while Ser41 received scores of 1 and 0.88 respectively. Scansite however, only detected all three sites on a low stringency scan, Phosphomotif finder detected only Ser33 as a putative site, and NetPhosK failed to detect any of the three sites. It seems therefore, that prediction of phosphorylation by GSK3β is not a simple procedure, and that the low scores obtained in this exercise should not be taken as definitive evidence that the PDE4s are not a direct target for GSK3β phosphorylation.

Therefore, further experimentation is required. This lies beyond the scope of this thesis, but is presently being planned in the Millar lab.

Figures 4.6A and B overleaf show aligned sequences of human PDE4B (A) and PDE4D (B) isoforms. As with all alignments performed by Clustal, a "*" depicts full conservation of an amino acid residue throughout isoforms, ":" depicts a conserved substitution, and "." A semi conserved substitution. Highlighted in green are the DISC1 PDE4 binding sites as defined in Murdoch *et al*, 2007. Highlighted in blue are known PKA and ERK phosphorylation sites, as defined by Mackenzie *et al* 2002, and Baillie *et al* 2000 respectively.

The key to highlighting throughout the figure is as follows:
S = identified as having phosphorylation potential in 2 databases
S = identified in 3 databases
S = identified in 2 databases, Scansite suggests GSK3α and β potential
= identified in 2 databases, Scansite suggests GSK3α only

Figure 4.6A


CLUSTAL 2.0.10 multiple sequence alignment
PDE4B3|NP_001032417.1 ---------MTAKDSSKELTASEPEVCIKTFKEQMHLELELPRLPGN--- 38
PDE4B5|ABQ85407.1| ----------
PDE4B2|NP_001032416.1 ----------
PDE4B1|NP_002591.2| MKKSRSVMTVMADDNVKDYFECSLSKSYSSSSNTLGIDLWRGRRCCSGNL 50
PDE4B3|NP_001032417.1 --RPTSPKISPRSSPRNSPCFFR-KLLVNKSIRQRRRFTVAHTCFDVENG 85
PDE4B5|ABQ85407.1| ----------
PDE4B2|NP_001032416.1 ----------
PDE4B1|NP_002591.2| QLPPLSQRQSERARTPEGDGISRPTTLPLTTLPSIAITTVSQECFDVENG 100
PKA
PDE4B3|NP_001032417.1 PSPGRSPLDPQASSSAGLVLHATFPGHSQRRESFLYRSDSDYDLSPKAMS 135
PDE4B5|ABQ85407.1| ----------
PDE4B2|NP_001032416.1 ----------MKEHGGTFSSTGIS 14
PDE4B1|NP_002591.2| PSPGRSPLDPQASSSAGLVLHATFPGHSQRRESFLYRSDSDYDLSPKAMS 150
PDE4B3|NP_001032417.1 RNSSLPSEQHGDDLIVTPFAQVLASLRSVRNNFTILTNLHGTSNKRSPAA 185
PDE4B5|ABQ85407.1| ----------MPEA 4
PDE4B2|NP_001032416.1 GGS-------GD--------SAMDSLQPLQPNYMPVCLFAEESYQKLAME 49
PDE4B1|NP_002591.2| RNSSLPSEQHGDDLIVTPFAQVLASLRSVRNNFTILTNLHGTSNKRSPAA 200
DISC1 Common
PDE4B3|NP_001032417.1 SQPPVSRVNPQEESYQKLAMETLEELDWCLDQLETIQTYRSVSEMASNKF 235
PDE4B5|ABQ85407.1| N----------YLLSVSWGYIKF 17
PDE4B2|NP_001032416.1 T----------LEELDWCLDQLETIQTYRSVSEMASNKF 78
PDE4B1|NP_002591.2| SQPPVSRVNPQEESYQKLAMETLEELDWCLDQLETIQTYRSVSEMASNKF 250

```
PDE4B3|NP_001032417.1      KRMLNRELTHLSEMSRSGNQVSEYISNTFLDKQNDVEIPSPTQKDREKKK 285
PDE4B5|ABQ85407.1|         KRMLNRELTHLSEMSRSGNQVSEYISNTFLDKQNDVEIPSPTQKDREKKK 67
PDE4B2|NP_001032416.1      KRMLNRELTHLSEMSRSGNQVSEYISNTFLDKQNDVEIPSPTQKDREKKK 128
PDE4B1|NP_002591.2|        KRMLNRELTHLSEMSRSGNQVSEYISNTFLDKQNDVEIPSPTQKDREKKK 300
                           **************************************************

PDE4B3|NP_001032417.1      KQQLMTQISGVKKLMHSSSLNNTSISRFGVNTENEDHLAKELEDLNKWGL 335
PDE4B5|ABQ85407.1|         KQQLMTQISGVKKLMHSSSLNNTSISRFGVNTENEDHLAKELEDLNKWGL 117
PDE4B2|NP_001032416.1      KQQLMTQISGVKKLMHSSSLNNTSISRFGVNTENEDHLAKELEDLNKWGL 178
PDE4B1|NP_002591.2|        KQQLMTQISGVKKLMHSSSLNNTSISRFGVNTENEDHLAKELEDLNKWGL 350
                           **************************************************
                                DISC1 PDE4B specific
PDE4B3|NP_001032417.1      NIFNVAGYSHNRPLTCIMYAIFQERDLLKTFRISSDTFITYMMTLEDHYH 385
PDE4B5|ABQ85407.1|         NIFNVAGYSHNRPLTCIMYAIFQERDLLKTFRISSDTFITYMMTLEDHYH 167
PDE4B2|NP_001032416.1      NIFNVAGYSHNRPLTCIMYAIFQERDLLKTFRISSDTFITYMMTLEDHYH 228
PDE4B1|NP_002591.2|        NIFNVAGYSHNRPLTCIMYAIFQERDLLKTFRISSDTFITYMMTLEDHYH 400
                           **************************************************

PDE4B3|NP_001032417.1      SDVAYHNSLHAADVAQSTHVLLSTPALDAVFTDLEILAAIFAAAIHDVDH 435
PDE4B5|ABQ85407.1|         SDVAYHNSLHAADVAQSTHVLLSTPALDAVFTDLEILAAIFAAAIHDVDH 217
PDE4B2|NP_001032416.1      SDVAYHNSLHAADVAQSTHVLLSTPALDAVFTDLEILAAIFAAAIHDVDH 278
PDE4B1|NP_002591.2|        SDVAYHNSLHAADVAQSTHVLLSTPALDAVFTDLEILAAIFAAAIHDVDH 450
                           **************************************************
                                                    DISC1 common
PDE4B3|NP_001032417.1      PGVSNQFLINTNSELALMYNDESVLENHHLAVGFKLLQEEHCDIFMNLTK 485
PDE4B5|ABQ85407.1|         PGVSNQFLINTNSELALMYNDESVLENHHLAVGFKLLQEEHCDIFMNLTK 267
PDE4B2|NP_001032416.1      PGVSNQFLINTNSELALMYNDESVLENHHLAVGFKLLQEEHCDIFMNLTK 328
PDE4B1|NP_002591.2|        PGVSNQFLINTNSELALMYNDESVLENHHLAVGFKLLQEEHCDIFMNLTK 500
                           **************************************************

PDE4B3|NP_001032417.1      KQRQTLRKMVIDMVLATDMSKHMSLLADLKTMVETKKVTSSGVLLLDNYT 535
PDE4B5|ABQ85407.1|         KQRQTLRKMVIDMVLATDMSKHMSLLADLKTMVETKKVTSSGVLLLDNYT 317
PDE4B2|NP_001032416.1      KQRQTLRKMVIDMVLATDMSKHMSLLADLKTMVETKKVTSSGVLLLDNYT 378
PDE4B1|NP_002591.2|        KQRQTLRKMVIDMVLATDMSKHMSLLADLKTMVETKKVTSSGVLLLDNYT 550
                           **************************************************
```

```
PDE4B3|NP_001032417.1      DRIQVLRNMVHCADLSNPTKSLELYRQWTDRIMEEFFQQGDKERERGMEI 585
PDE4B5|ABQ85407.1|         DRIQVLRNMVHCADLSNPTKSLELYRQWTDRIMEEFFQQGDKERERGMEI 367
PDE4B2|NP_001032416.1      DRIQVLRNMVHCADLSNPTKSLELYRQWTDRIMEEFFQQGDKERERGMEI 428
PDE4B1|NP_002591.2|        DRIQVLRNMVHCADLSNPTKSLELYRQWTDRIMEEFFQQGDKERERGMEI 600
                           ************************************************

PDE4B3|NP_001032417.1      SPMCDKHTASVEKSQVGFIDYIVHPLWETWADLVQPDAQDILDTLEDNRN 635
PDE4B5|ABQ85407.1|         SPMCDKHTASVEKSQVGFIDYIVHPLWETWADLVQPDAQDILDTLEDNRN 417
PDE4B2|NP_001032416.1      SPMCDKHTASVEKSQVGFIDYIVHPLWETWADLVQPDAQDILDTLEDNRN 478
PDE4B1|NP_002591.2|        SPMCDKHTASVEKSQVGFIDYIVHPLWETWADLVQPDAQDILDTLEDNRN 650
                           ************************************************
                                 ERK
PDE4B3|NP_001032417.1      WYQSMIPQSPSPPLDEQNRDCQGLMEKFQFELTLDEEDSEGPEKEGEGHS 685
PDE4B5|ABQ85407.1|         WYQSMIPQSPSPPLDEQNRDCQGLMEKFQFELTLDEEDSEGPEKEGEGHS 467
PDE4B2|NP_001032416.1      WYQSMIPQSPSPPLDEQNRDCQGLMEKFQFELTLDEEDSEGPEKEGEGHS 528
PDE4B1|NP_002591.2|        WYQSMIPQSPSPPLDEQNRDCQGLMEKFQFELTLDEEDSEGPEKEGEGHS 700
                           ************************************************

PDE4B3|NP_001032417.1      YFSSTKTLCVIDPENRDSLGETDIDIATEDKSPVDT 721
PDE4B5|ABQ85407.1|         YFSSTKTLCVIDPENRDSLGETDIDIATEDKSPVDT 503
PDE4B2|NP_001032416.1      YFSSTKTLCVIDPENRDSLGETDIDIATEDKSPVDT 564
PDE4B1|NP_002591.2|        YFSSTKTLCVIDPENRDSLGETDIDIATEDKSPVDT 736
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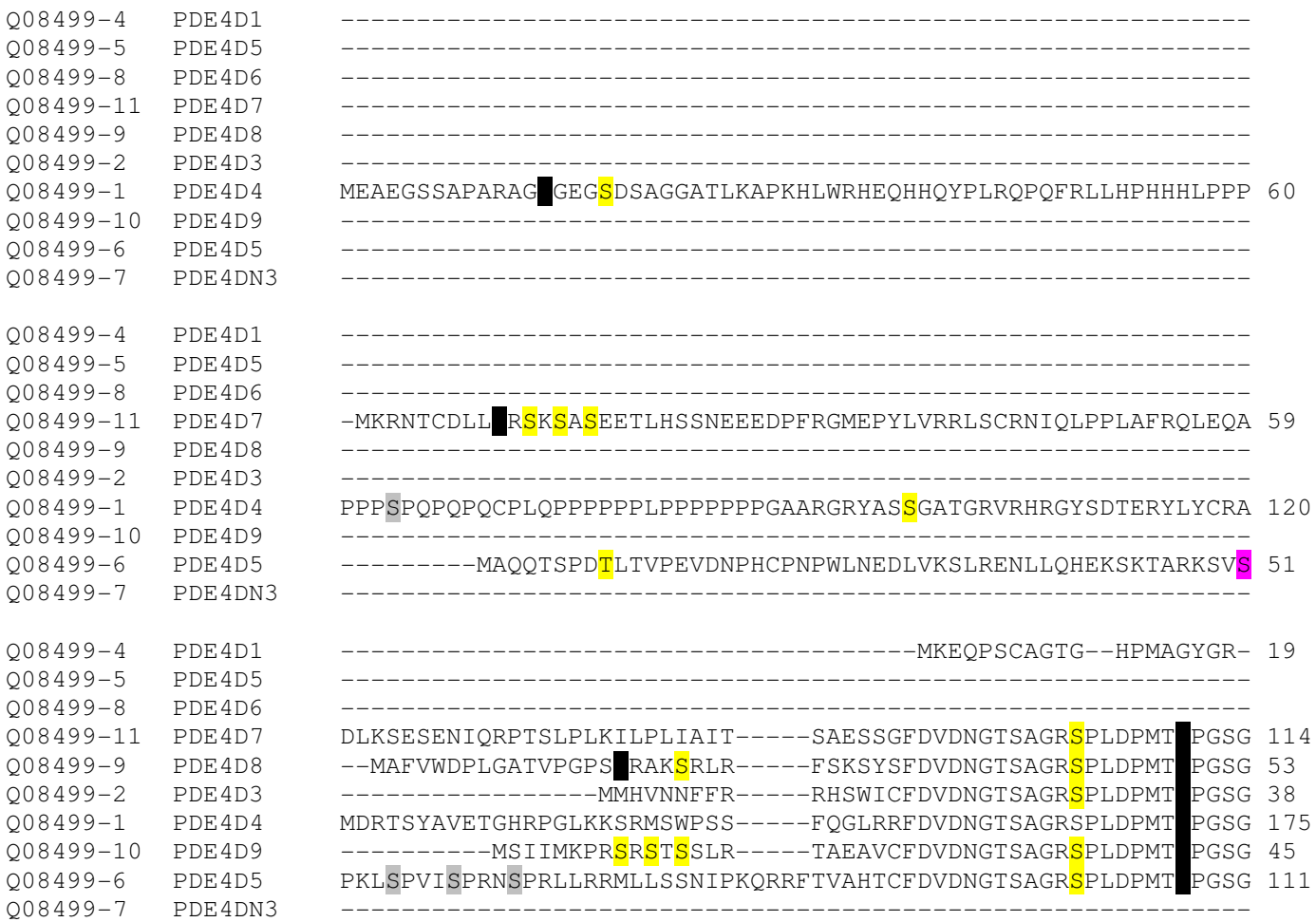
```

Figure 4.6B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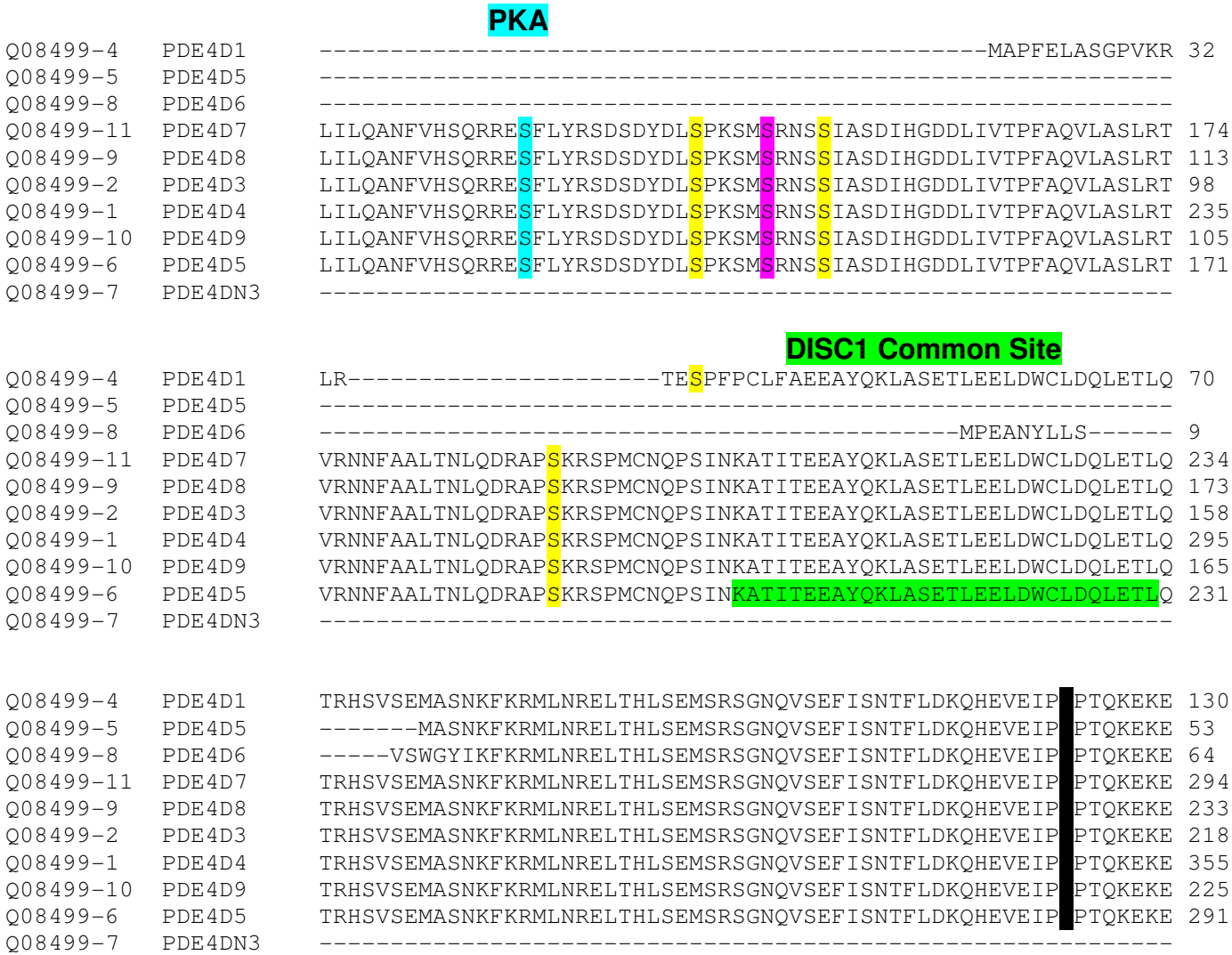
Q08499-10 PDE4D9 ----------
Q08499-6 PDE4D5 ----------
Q08499-7 PDE4DN3 ----------
Q08499-4 PDE4D1 ----------
Q08499-5 PDE4D5 ----------
Q08499-8 PDE4D6 ----------
Q08499-11 PDE4D7 -MKRNTCDLL RSKSASEETLHSSNEEEDPFRGMEPYLVRRLSCRNIQLPPLAFRQLEQA 59
Q08499-9 PDE4D8 ----------
Q08499-2 PDE4D3 ----------
Q08499-1 PDE4D4 PPPSPQPQPQCPLQPPPPPPLPPPPPPPGAARGRYASSGATGRVRHRGYSDTERYLYCRA 120
Q08499-10 PDE4D9 ----------
Q08499-6 PDE4D5 ---------MAQQTSPDTLTVPEVDNPHCPNPWLNEDLVKSLRENLLQHEKSKTARKSVS 51
Q08499-7 PDE4DN3 ----------
Q08499-4 PDE4D1 ----------MKEQPSCAGTG--HPMAGYGR- 19
Q08499-5 PDE4D5 ----------
Q08499-8 PDE4D6 ----------
Q08499-11 PDE4D7 DLKSESENIQRPTSLPLKILPLIAIT-----SAESSGFDVDNGTSAGRSPLDPMT PGSG 114
Q08499-9 PDE4D8 --MAFVWDPLGATVPGPS RAKSRLR-----FSKSYSFDVDNGTSAGRSPLDPMT PGSG 53
Q08499-2 PDE4D3 ----------MMHVNNFFR-----RHSWICFDVDNGTSAGRSPLDPMT PGSG 38
Q08499-1 PDE4D4 MDRTSYAVETGHRPGLKKSRMSWPSS-----FQGLRRFDVDNGTSAGRSPLDPMT PGSG 175
Q08499-10 PDE4D9 ----------MSIIMKPRSRSTSSLR-----TAEAVCFDVDNGTSAGRSPLDPMT PGSG 45
Q08499-6 PDE4D5 PKLSPVISPRNSPRLLRRMLLSSNIPKQRRFTVAHTCFDVDNGTSAGRSPLDPMT PGSG 111
Q08499-7 PDE4DN3 ----------


PKA
DISC1 Common Site
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Q08499-8 PDE4D6 ----------
Q08499-11 PDE4D7 LILQANFVHSQRRESFLYRSDSDYDLSPKSMSRNSSIASDIHGDDLIVTPFAQVLASLRT 174
Q08499-9 PDE4D8 LILQANFVHSQRRESFLYRSDSDYDLSPKSMSRNSSIASDIHGDDLIVTPFAQVLASLRT 113
Q08499-2 PDE4D3 LILQANFVHSQRRESFLYRSDSDYDLSPKSMSRNSSIASDIHGDDLIVTPFAQVLASLRT 98
Q08499-1 PDE4D4 LILQANFVHSQRRESFLYRSDSDYDLSPKSMSRNSSIASDIHGDDLIVTPFAQVLASLRT 235
Q08499-10 PDE4D9 LILQANFVHSQRRESFLYRSDSDYDLSPKSMSRNSSIASDIHGDDLIVTPFAQVLASLRT 105
Q08499-6 PDE4D5 LILQANFVHSQRRESFLYRSDSDYDLSPKSMSRNSSIASDIHGDDLIVTPFAQVLASLRT 171
Q08499-7 PDE4DN3 ----------
Q08499-4 PDE4D1 LR----------TESPFPCLFAEEAYQKLASETLEELDWCLDQLETLQ 70
Q08499-5 PDE4D5 ----------
Q08499-8 PDE4D6 ----------MPEANYLLS------ 9
Q08499-11 PDE4D7 VRNNFAALTNLQDRAPSKRSPMCNQPSINKATITEEAYQKLASETLEELDWCLDQLETLQ 234
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Q08499-1 PDE4D4 VRNNFAALTNLQDRAPSKRSPMCNQPSINKATITEEAYQKLASETLEELDWCLDQLETLQ 295
Q08499-10 PDE4D9 VRNNFAALTNLQDRAPSKRSPMCNQPSINKATITEEAYQKLASETLEELDWCLDQLETLQ 165
Q08499-6 PDE4D5 VRNNFAALTNLQDRAPSKRSPMCNQPSINKATITEEAYQKLASETLEELDWCLDQLETLQ 231
Q08499-7 PDE4DN3 ----------
Q08499-4 PDE4D1 TRHSVSEMASNKFKRMLNRELTHLSEMSRSGNQVSEFISNTFLDKQHEVEIPPTQKEKE 130
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Q08499-8 PDE4D6 -----VSWGYIKFKRMLNRELTHLSEMSRSGNQVSEFISNTFLDKQHEVEIPPTQKEKE 64
Q08499-11 PDE4D7 TRHSVSEMASNKFKRMLNRELTHLSEMSRSGNQVSEFISNTFLDKQHEVEIPPTQKEKE 294
Q08499-9 PDE4D8 TRHSVSEMASNKFKRMLNRELTHLSEMSRSGNQVSEFISNTFLDKQHEVEIPPTQKEKE 233
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Q08499-10 PDE4D9 TRHSVSEMASNKFKRMLNRELTHLSEMSRSGNQVSEFISNTFLDKQHEVEIPPTQKEKE 225
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Q08499-11   PDE4D7    KKKRPMSQISGVKKLMHSSSLTNSSIPRFGVKTEQEDVLAKELEDVNKWGLHVFRIAELS 354
Q08499-9    PDE4D8    KKKRPMSQISGVKKLMHSSSLTNSSIPRFGVKTEQEDVLAKELEDVNKWGLHVFRIAELS 293
Q08499-2    PDE4D3    KKKRPMSQISGVKKLMHSSSLTNSSIPRFGVKTEQEDVLAKELEDVNKWGLHVFRIAELS 278
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Q08499-10   PDE4D9    KKKRPMSQISGVKKLMHSSSLTNSSIPRFGVKTEQEDVLAKELEDVNKWGLHVFRIAELS 285
Q08499-6    PDE4D5    KKKRPMSQISGVKKLMHSSSLTNSSIPRFGVKTEQEDVLAKELEDVNKWGLHVFRIAELS 351
Q08499-7    PDE4DN3   -----MAQQTSPDTLTVP-EVDNPHCP------------------NPW----------- 24
                           *:* :. ..*  . .: *.  *                  * *

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                                  :.**:*:::          .* :* ::..* :.

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                                       ::. ::..*   : * : .::*:.:*
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Q08499-8    PDE4D6    LAVGFKLLQEENCDIFQNLTKKQRQSLRKMVIDIVLATDMSKHMNLLADLKTMVETKKVT 304
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Q08499-2    PDE4D3    SSGVLLLDNYSDRIQVLQNMVHCADLSNPTKPLQLYRQWTDRIMEEFFRQGDRERERGME 518
Q08499-1    PDE4D4    SSGVLLLDNYSDRIQVLQNMVHCADLSNPTKPLQLYRQWTDRIMEEFFRQGDRERERGME 655
Q08499-10   PDE4D9    SSGVLLLDNYSDRIQVLQNMVHCADLSNPTKPLQLYRQWTDRIMEEFFRQGDRERERGME 525
Q08499-6    PDE4D5    SSGVLLLDNYSDRIQVLQNMVHCADLSNPTKPLQLYRQWTDRIMEEFFRQGDRERERGME 591
Q08499-7    PDE4DN3   GSGLILQAN----------FVHSQRR------------------ESFLYRSDSD----YD 136
                      .**::*  *          :**.                    *.*: :.* :     :
                                                 DISC1 PDE4D Specific     ERK
Q08499-4    PDE4D1    ISPMCDKHNASVEKSQVGFIDYIVHPLWETWADLVHPDAQDILDTLEDNREWYQSTIPQ  490
Q08499-5    PDE4D5    ISPMCDKHNASVEKSQVGFIDYIVHPLWETWADLVHPDAQDILDTLEDNREWYQSTIPQ  413
Q08499-8    PDE4D6    ISPMCDKHNASVEKSQVGFIDYIVHPLWETWADLVHPDAQDILDTLEDNREWYQSTIPQ  424
Q08499-11   PDE4D7    ISPMCDKHNASVEKSQVGFIDYIVHPLWETWADLVHPDAQDILDTLEDNREWYQSTIPQ  654
Q08499-9    PDE4D8    ISPMCDKHNASVEKSQVGFIDYIVHPLWETWADLVHPDAQDILDTLEDNREWYQSTIPQ  593
Q08499-2    PDE4D3    ISPMCDKHNASVEKSQVGFIDYIVHPLWETWADLVHPDAQDILDTLEDNREWYQSTIPQ  578
Q08499-1    PDE4D4    ISPMCDKHNASVEKSQVGFIDYIVHPLWETWADLVHPDAQDILDTLEDNREWYQSTIPQ  715
Q08499-10   PDE4D9    ISPMCDKHNASVEKSQVGFIDYIVHPLWETWADLVHPDAQDILDTLEDNREWYQSTIPQ  585
Q08499-6    PDE4D5    ISPMCDKHNASVEKSQVGFIDYIVHPLWETWADLVHPDAQDILDTLEDNREWYQSTIPQ  651
Q08499-7    PDE4DN3   LSPKSMSRNSSIA-SDIHGDDLIVTPFAQVLASLR---------TVRNN--FAALTNLQD 184
                      :** . .:*:*:  *::   * ** *: :. *.*          *:.:*  :   *  *.
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Q08499-4    PDE4D1    P PAPDDPEEGRQGQTEKFQFELTLEEDGESDTEKDSGSQVEEDTSCSDSKTLCTQDSES 550
Q08499-5    PDE4D5    P PAPDDPEEGRQGQTEKFQFELTLEEDGESDTEKDSGSQVEEDTSCSDSKTLCTQDSES 473
Q08499-8    PDE4D6    P PAPDDPEEGRQGQTEKFQFELTLEEDGESDTEKDSGSQVEEDTSCSDSKTLCTQDSES 484
Q08499-11   PDE4D7    P PAPDDPEEGRQGQTEKFQFELTLEEDGESDTEKDSGSQVEEDTSCSDSKTLCTQDSES 714
Q08499-9    PDE4D8    P PAPDDPEEGRQGQTEKFQFELTLEEDGESDTEKDSGSQVEEDTSCSDSKTLCTQDSES 653
Q08499-2    PDE4D3    P PAPDDPEEGRQGQTEKFQFELTLEEDGESDTEKDSGSQVEEDTSCSDSKTLCTQDSES 638
Q08499-1    PDE4D4    P PAPDDPEEGRQGQTEKFQFELTLEEDGESDTEKDSGSQVEEDTSCSDSKTLCTQDSES 775
Q08499-10   PDE4D9    P PAPDDPEEGRQGQTEKFQFELTLEEDGESDTEKDSGSQVEEDTSCSDSKTLCTQDSES 645
Q08499-6    PDE4D5    P PAPDDPEEGRQGQTEKFQFELTLEEDGESDTEKDSGSQVEEDTSCSDSKTLCTQDSES 711
Q08499-7    PDE4DN3   RAPSKRSP-------------------------------------------MCNQPS-- 198
                       :*:  .*                                          :*.* *

Q08499-4    PDE4D1    TEIPLDEQVEEEAVGEEEESQPEACVIDDRSPDT 584
Q08499-5    PDE4D5    TEIPLDEQVEEEAVGEEEESQPEACVIDDRSPDT 507
Q08499-8    PDE4D6    TEIPLDEQVEEEAVGEEEESQPEACVIDDRSPDT 518
Q08499-11   PDE4D7    TEIPLDEQVEEEAVGEEEESQPEACVIDDRSPDT 748
Q08499-9    PDE4D8    TEIPLDEQVEEEAVGEEEESQPEACVIDDRSPDT 687
Q08499-2    PDE4D3    TEIPLDEQVEEEAVGEEEESQPEACVIDDRSPDT 672
Q08499-1    PDE4D4    TEIPLDEQVEEEAVGEEEESQPEACVIDDRSPDT 809
Q08499-10   PDE4D9    TEIPLDEQVEEEAVGEEEESQPEACVIDDRSPDT 679
Q08499-6    PDE4D5    TEIPLDEQVEEEAVGEEEESQPEACVIDDRSPDT 745
Q08499-7    PDE4DN3   --------INKATITGLYNGIIAFL--------- 215
                              ::: ::
```

Figure 4.6C

Position	Residue	DP 1.3	Scansite	KP 2.0	PM Finder	NPK
6	S	0.50		0.52		
56	S		3A		Yes	
65	T	0.50			Yes	
76	T		3A	0.56		
102	S	0.54	3A		Yes	0.54
106	S	0.60	3A		Yes	0.54
145	S	0.64			Yes	
150	S	0.58		0.78	Yes	
154	S	0.55		0.65		
197	S				Yes	0.52
290	S	0.59		0.56	Yes	0.51
424	T			0.62	Yes	
601	S				Yes	0.5
610	S			0.65	Yes	
718	S	0.51		0.62		

Figure 4.6D

Position	Residue	DP 1.3	Scansite	KP 2.0	PM finder	NPK
14	S	0.935	*3A*		Yes	0.51
18	S	0.68		0.767		
64	S	0.827	**3A**		Yes	0.53
97	S	0.75		0.7555		
142	S	0.643			Yes	
164	S				Yes	0.51
171	S		*3A*		Yes	0.51
202	S	0.655			Yes	
207	S	0.56		0.5608	Yes	
211	S	0.509		0.547		
252	S	0.512		0.792		
348	S	0.612	*3A*	0.555	Yes	0.5
480	T			0.6288	Yes	
657	S				Yes	0.5
666	S			0.7137	Yes	
715	S	0.621	3A		Yes	0.51
717	S	0.606	*3A*	0.6192		
754	S	0.89		0.5308		
761	S	0.747			Yes	
806	S	0.754			Yes	0.5

Figure 4.6C-D (previous page): Tables showing the potential phosphorylation sites depicted on the sequence alignments of PDE4B (C) and PDE4D (D), and the scores obtained from each phosphorylation database. In Disphos 1.3 (DP 1.3), KinasePhos 2.0 (KP2.0) and NetPhosK (NPK), the score roughly approximates to the probability of phosphorylation, and so a score of 0 implies there in no probability of phosphorylation, and a score of 1 implies phosphorylation will definitely occur. 0.5 is the threshold value for reporting a site. In the Scansite column, if the site is recognised by a high stringency scan, it is depicted in bold, if medium stringency in normal type, and by low stringency in italics. Phosphomotif finder (PM Finder) gives a binary response for a particular kinase, if it detected a site, yes was entered into the appropriate column.

4.3 Discussion

In this chapter I have confirmed the validity of the SHSY5Y system as a model for assessing PDE4 activity. SHSY5Y cells produced a PDE4 specific 1.4 fold increase in activity in response to forskolin, almost identical to that observed in the previous literature by over expressed PDE4B1 in COS7 cells (MacKenzie et al., 2002). This response has been shown to be wholly due to phosphorylation of PDE4 long forms by PKA, as the response was obliterated by the use of the specific PKA inhibitor H89, and by site directed mutagenesis of the PKA phosphorylation site corresponding to residue 133 in PDE4B1. Thus, forskolin activates PKA signalling by switching on adenylate cyclase, PKA phosphorylates long forms of PDE4, and a measurable increase in PDE4 activity is observed (MacKenzie et al., 2002). qPCR with pan probes for PDE4 isoforms in Chapter 3 detected the presence of PDE4A, B and D in SHSY5Y cells, but not PDE4C.

GSK3β is an enzyme that has been strongly implicated in psychiatric disease both by virtue of its inhibition by the mood stabilising drug lithium, and genetic and pharmacological experiments reviewed in the Introduction. Over 40 putative downstream targets for GSK3β phosphorylation have been described, and the enzyme itself has an exquisitely complex and multi tiered regulatory system (Jope and Johnson, 2004). GSK3β itself is phosphorylated by kinases from a variety of different signalling pathways, including the structurally similar AKT and PKA, both of which phosphorylate Ser9 to inhibit GSK3β activity (Cross et al., 1995, Fang et al., 2000). The RII subunit of PKA is in turn phosphorylated by GSK3β (Brian et al., 1982). This enables integration of multiple signalling pathways within the cell to produce very fine control of output. Further to this level of regulation, the presence or absence of proteins in a binding complex (Ikeda et al., 1998), and the presence of pre phosphorylated "primed" residues can effect the affinity of GSK3β for a substrate (Fiol et al., 1987, Thomas G, 1999), allowing even finer contextual control of enzyme activity.

It has recently been shown that DISC1 binds to GSK3β in foetal mouse brain lysate (Mao et al., 2009), which has raised the interesting idea of the formation of a complex between GSK3β, DISC1, and PDE4B/D. The formation of such a complex could have implications for the activity of both GSK3β and the PDE4s, with DISC1 acting as a scaffold bringing other interacting proteins together and altering substrate specificity of GSK3β. To this end, I performed simple pharmacological manipulations to try and ascertain whether GSK3β has an effect on PDE4 activity.

I used the AlphaScreen assay system to assess cAMP accumulation in SHSY5Y cells in response to LiCl treatment. cAMP accumulation increased significantly at high 10mM concentrations of LiCl treatment, corresponding to five times the IC_{50} of LiCl for GSK3β. The IC_{50} is the concentration required to inhibit 50% of a biological process, thus it is likely that at 10mM the system is approaching complete inhibition of GSK3β. The AlphaScreen signal decreased by 15% (+/-1.17), corresponding to an equivalent increase in cAMP concentration. A corresponding decrease in PDE4 specific activity of

24% (=/-2.4) was observed in low throughput PDE assays. No significant changes were observed in non-PDE4 activity, although this component of the response forms a small minority of the activity measured. To assess whether the observed changes were due to inhibition of GSK3β by LiCl, the same assay was performed using 3μM SB216763, a GSK3β specific inhibitor with an IC_{50} of 34nM (Coghlan et al., 2000). In this series of experiments, 10mM LiCl treatment caused an 18% (+/-2.6) decrease in PDE4 activity, while 3μM SB216763 resulted in a 13% (+/-1.1) decrease that was not statistically different by t-test to the effect produced by LiCl. There was a small trend towards an additive effect of using both compounds, but again this was not statistically significant. Thus it is apparent that the changes in PDE4 activity due to LiCl treatment are due to the effect of LiCl in inhibiting GSK3β. SB216763 has minimal effects at other cyclin dependant kinases, but LiCl does not inhibit these same kinases, and thus they are ruled out as a responsible pathway (Cohen and Goedert, 2004).

10mM treatment of cell culture with LiCl is above the generally accepted "therapeutic threshold," of lithium treatment, which is a blood plasma concentration of between 0.6-1.2mM (Jope and Song, 1997). It is difficult to find reports in the literature of whether there is potential for accumulation of lithium in particular compartments of the body. Mechanisms for amplifying the effects of lower concentrations of lithium however, have been described. Firstly, GSK3β is very highly expressed in brain tissue (Woodgett, 1990). Competition with magnesium ions is the mechanism by which lithium exerts its effect (Ryves and Harwood, 2001), and magnesium concentration within the brain is very tightly regulated: infusing high concentrations of magnesium into the plasma does not alter brain magnesium levels in swine (Gee et al., 2001). The mechanism of action infers that lithium treatment will have a greater effect where free tissue magnesium is low. In the rat brain, total tissue magnesium concentration is 6.2μmoles/g, lower than the concentration in liver and kidney. Free magnesium however, does not vary between these three tissues, lying between 0.6-1.3μM/g (Veloso et al., 1973). At these concentrations of magnesium, the IC_{50} of lithium may be decreased to 0.8mM, clearly allowing for significant inhibition within the therapeutic index (Ryves and Harwood,

2001). Finally, increased inhibitory phosphorylation of Ser9 of GSK3β has been shown in SHSY5Y cells in response to medium term lithium treatment. Phosphorylation of the residue increased over the course of the 24 hour experiment. There was no concomitant change in AKT activation by phosphorylation, and so this adaptive change is thought to be independent of AKT signalling. Increased Ser9 phosphorylation was also demonstrated *in vivo*, with an increase observed in mouse cortex and hippocampus after 4 weeks of treatment resulting in serum concentrations of 0.7mM lithium (De Sarno et al., 2002). These factors could all lead to the potentiation of the effects of lithium in brain tissue after medium to long term treatment *in vivo*.

The potential for PDE4s to be directly phosphorylated by GSK3β was briefly investigated through the use of phosphorylation databases. The most stringent of these, Scansite, was only able to detect putative GSK3β sites in PDE4B3, D5 and DN3, and these sites were only suggested on a low stringency scan, ranking just under halfway down the list of total possible GSK3β sites in the vertebrate section of the SwissProt database. Further clusters of low scoring putative sites were highlighted by the other non GSK3β specific databases, but none of these stood out as obvious candidates for further study such as site directed mutagenesis. GSK3β however, is not a typical kinase enzyme. To phosphorylate a majority of substrates it prefers the substrate to be primed by phosphorylation four residues away from the GSK3β site. The affinity for a substrate can also be altered by the presence or absence of other proteins from the complex binding to it (Frame and Cohen, 2001, Jope and Johnson, 2004). When the sequence of β-catenin, a protein with a cluster of well characterised GSK3β phosphorylation sites was run through the same prediction tools, three of them either failed to detect these established sites, or only detected them in low stringency scans. This tendency towards non canonical behaviour suggests that the presence of no clear canonical GSK3β site in the phosphorylation databases should not totally rule out the potential for direct phosphorylation of PDE4s by GSK3β.

Further investigation however, is complicated by the tendency of GSK3β to require priming phosphorylation. Without priming, the efficiency of phosphorylation is seriously reduced. There are no putative GSK3β sites four residues distant from established PKA and ERK phosphorylation sites (Figures 4.6A and B). This makes *in vitro* phosphorylation assays involving direct mixing of recombinant protein and kinase difficult. Should another kinase be added to the mix? If so, which one? It may also be possible to drive a phosphorylation by using a very high concentration of purified GSK3β. From the phosphorylation prediction tools, there is no clear or even leading candidate for functional studies involving mutagenesis. It is likely that a higher throughput approach to phosphorylation detection is required, such as mass spectrometry (Mann et al., 2002) or the use of microarray technology. Recent advances in technology allow immobilisation of small peptide sequences onto assay chips, which can then be probed with the kinase of interest. "Hits" can be detected by incubation with radioactively labeled ATP, phosphospecific antibodies or phosphopeptide chelators attached to a label such as a fluorescent dye or horse radish peroxidase (Henderson and Bradley, 2007, Schutkowski M et al., 2005). Again, this fails to get round the need for substrate priming, and there are also arguments about whether such small peptide fragments can mimic kinase behaviour *in vivo*, due to their lack of complex tertiary structure. Further investigation of the potential for direct phosphorylation was thus outside the capabilities of this project, but not ruled out by initial work.

The possibility that basal activity of PDE4s is maintained by GSK3β is tantalising. The further means for integration of PKA and cAMP signalling with GSK3β and AKT pathways would allow for even finer contextual control of exquisitely sensitive signalling systems, allowing the tuning of the response of an enzyme with a large number of substrates to very specific cellular situations. The dynamic binding of DISC1 to PDE4s and GSK3β also suggests a role for DISC1 in this integration of signals, and this will be investigated in Chapter 5.

5 The effect of DISC1 on PDE4 activity – does it alter responses to PKA and GSK3β signalling?

5.1 Introduction

The dynamic and isoform specific interaction between DISC1 and the PDE4s is one of the most well characterised and established interactions of DISC1. As a brief summary, in transfected HEK cells, PKA dependent phosphorylation of PDE4D3 and PDE4C2 results in dissociation of 100kDa DISC1. Forskolin stimulation has no effect on the binding of PDE4A5 or PDE4B1 to 100kDa DISC1 (Murdoch et al., 2007). This is in contrast to the interaction observed with the 71kDa isoform of endogenous DISC1, which is released from PDE4B upon activation by PKA in SHSY5Y cells (Millar et al., 2005b). The interaction between DISC1 and GSK3β is less well established (Mao et al., 2009), indeed, unpublished work by Fumiaki Ogawa has shown that DISC1 and GSK3β interact only weakly when both are exogenously expressed in COS7 cells. This weak interaction appears to stabilise if a putative GSK3β phosphorylation site on DISC1, S396A, is mutated to an alanine residue (Ogawa *et al*, paper in preparation). DISC1 interaction with both PDE4 and GSK3β therefore raises the possibility of a highly dynamic intracellular signalling complex being formed, with the scaffolding protein DISC1 having the potential to alter the outcome of a signalling event by direct binding to PDE4s or GSK3β, or by bringing other interactors into close proximity with these and/or other signalling molecules.

PDE4B, C and D isoforms are phosphorylated at their C terminus by ERK2. This event inhibits the activity of long forms of the PDE4s, and activates the short form PDE4B2 (Baillie et al., 2000). DISC1 is already believed to play a role in ERK and AKT signalling (Hashimoto et al., 2006, Kim et al., 2009b, Enomoto et al., 2009, Shinoda et al., 2007). A 70% reduction in DISC1 expression in 7 D.I.V primary cortical neuron cultures leads to decreased activating phosphorylation of ERK1/2 and AKT.

Conversely, overexpression of DISC1 leads to increased levels of pERK1/2. When DISC1 containing either the serine and cysteine form of the SNP at residue 704 is overexpressed, only the serine variant caused a significant increase in pAKT levels. Rescue experiments with the cysteine form of DISC1 returned phosphorylation levels of pAKT and pERK1/2 to those of control cells, while neurons rescued with the serine form "overshot" the baseline control measurement (Hashimoto et al., 2006). An alternate study however found that DISC1 knockdown did not alter ERK phosphorylation when PC12 cells were stimulated by NGF treatment (Shinoda et al., 2007). They did observe reduction of pERK1/2 at growth cones in hippocampal neurons with DISC1 knockdown, but not in the cell bodies. ERK1/2 phosphorylation was restored by DISC1 overexpression in a successful rescue experiment (Shinoda et al., 2007).

As previously alluded to in Chapter 4, changes in DISC1 expression can also have effects on AKT signalling. Two groups have shown direct binding of KIAA1212 to DISC1, though there is controversy as to the regions involved in the interaction (Kim et al., 2009b, Enomoto et al., 2009). This interaction appears to modulate KIAA1212 (also known as Girdin and AKT phosphorylation enhanced, APE), a protein highly expressed during neuronal differentiation which directly binds and activates AKT. DISC1 knockdown in primary hippocampal neurons leads to increased levels of phosphor AKT (Kim et al., 2009b) and aberrant localisation of KIAA1212 (Enomoto et al., 2009). As alluded to previously, AKT signalling feeds into GSK3β signalling via inhibitory phosphorylation of Ser9 of GSK3β (Cross et al., 1995). Despite this, no effect was reported of DISC1 knockdown on Ser9 phosphorylation in adult hippocampal progenitor cells (Mao et al., 2009).

If one brings together this knowledge about the binding partners of DISC1, the multi-tiered levels of control over the activity of GSK3β, and the finding in the previous chapter that GSK3β affects the activity of PDE4s, it is reasonable to hypothesise that a multi-component signalling complex exists. The components of this complex may be

dynamically recruited and released in response to specific stimuli, and in different subcellular locations. Thus, this complex may act as a point of integration for external stimuli and internal signalling pathways. The context in which the complex finds itself, the proteins present within it and the phosphorylation states of these proteins are key to the output, be this the activation of further signalling pathways, the switching off or on of cellular processes, or changes in the activity of the enzymes involved, such as PDE4. It is certainly possible, given its function as a scaffold with the potential for binding multiple proteins, that the presence or absence of DISC1 may significantly alter the output of these complexes. I have attempted to address this possibility with a series of simple pharmacological manipulations and phosphodiesterase activity assays of the SHSY5Y based stable cells lines described earlier.

5.2 Results

5.2.1 Short term overexpression of DISC1 inhibits the increase in PDE4 activity in response to forskolin treatment

DISC1 overexpression was induced in the DISC1 inducible stable cell line using 1µg/ml tetracycline for 24 hours. This SHSY5Y based cell line overexpresses 100kDa DISC1 in response to application of tetracycline. For the final 4 hours of this treatment, cells were also serum starved. Cells were then treated for 30 minutes with vehicle or 10µM forskolin, and PDE activity assayed as described previously. There was no significant change in baseline PDE4 activity with respect to the induction status of DISC1. In uninduced cells, a 22% (+/-7) increase in activity occurred on treatment with forskolin. When DISC1 was induced, this increase was not observed: PDE4 activity remained at baseline levels (Figure 5.1A).

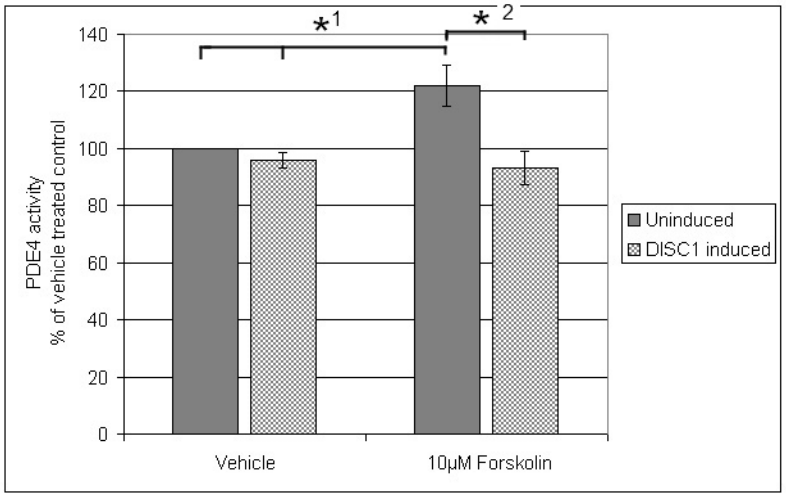


Figure 5.1A: Graph showing the effect of 10μM forskolin treatment on PDE4 activity in DISC1 inducible SHSY5Y cells. The effect of forskolin treatment in uninduced cells is clearly significant (*1, n=4, p=0.02). When DISC1 is induced, PDE4 activity remains at baseline: *2, n=4, p=0.02. Mean PDE4 activity in the vehicle treated control is 111.8 pmoles/mg/min.

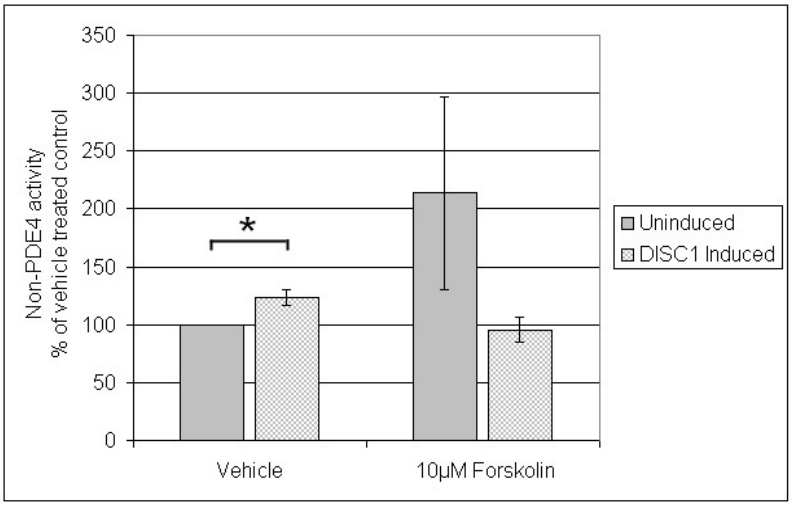


Figure 5.1B: Graph showing the effect of 10μM forskolin treatment on non-PDE4 activity in DISC1 inducible SHSY5Y cells. Non-PDE4 activity is a small component of the measurable activity (20%), and the only change that reaches significance is the increase in baseline non-PDE4 activity. *n=4, p=0.01. Mean non-PDE4 activity in the vehicle treated control is 23.9 pmoles/mg/min.

In these same treated cells, there is a trend to a similar response in non-PDE4 activity, but none of these changes are significant. There is however, a significant increase in the baseline of non-PDE4 activity when DISC1 is induced, perhaps suggesting that a mechanism operates to compensate for effects of DISC1 on PDE4 (Figure 5.1B).

Western blotting was performed with the α-DISC1 antibody to ensure that overexpression of 100kDa DISC1 occurred in response to tetracycline treatment as expected. At a short exposure time of 30 seconds overexpression of DISC1 was clearly visible. Two sample Western blots from this experiment are shown in Figure 5.2A. As a comparison, a blot from the same cells is shown at 2.5 minutes exposure, to show endogenous DISC1 staining beginning to appear (Figure 5.2B)

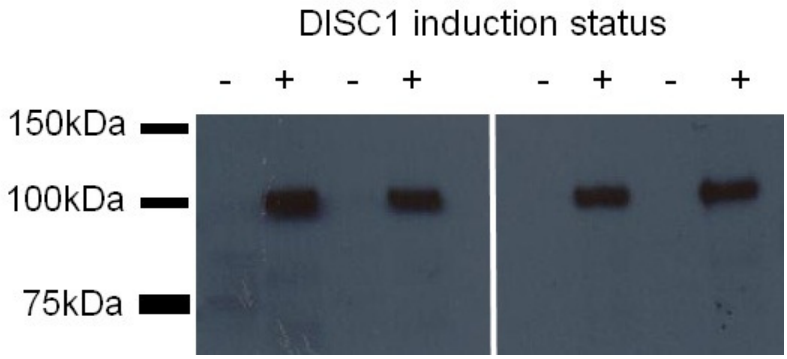


Figure 5.2A: Sample western blot of two experiments from 5.2.1. At a short exposure time of 30 seconds, overexpression of DISC1 is clearly visible in induced cells. An exposure time of over 2 minutes is required to visualise endogenous protein.

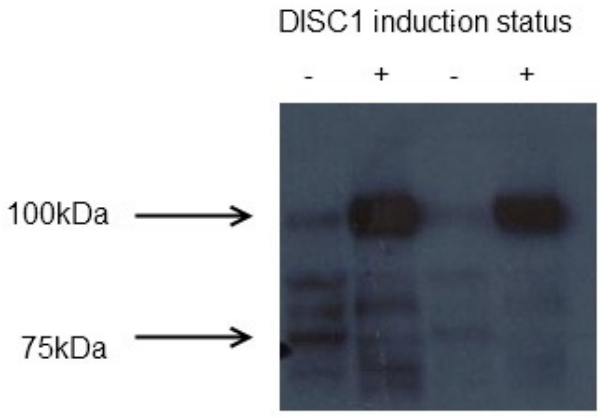


Figure 5.2B: A blot from the same samples as 5.2A after 2.5 minutes of exposure to film. The signal from endogenous DISC1 is beginning to appear, with strong bands at 100kDa and 75kDa.

5.2.2 Constitutive knockdown of DISC1 inhibits the forskolin stimulated increase in PDE4 activity

The stable SHSY5Y lines constitutively expressing T0 (control scramble shRNA) and T1 (DISC1 knockdown) were serum starved overnight and subjected to 30 minutes of treatment with 10μM forskolin, then PDE activity assayed as previously described. No significant difference in basal unstimulated PDE4 activity was observed on DISC1 knockdown (Figure 5.3A). A significant increase in PDE4 activity was observed in both control and knock down cell lines in response to forskolin treatment (n=7, p=0.000003, p=0.04 respectively, Figure 5.3A). The PDE4 activity increase observed in control scramble shRNA expressing cells fell within the range of activity previously defined in wild type SHSY5Y cells (Figure 4.1), demonstrating that there was no effect of shRNA expression on PDE4 activity in response to forskolin. In the DISC1 knockdown cell line, the magnitude of the increase in PDE4 activity following forskolin treatment was significantly smaller (n=7, p=0.02), from an average of 34% (+/-4) in the control line to 17% (+/-5) in the knockdown line (Figure 5.3B). Once again, none of the changes observed in the non-PDE4 component of the response were significant (Figure 5.3C).

Western blot analysis was performed to ensure that DISC1 was being knocked down in the T1 cells as expected. After 10 minutes exposure, a clear signal was obtained from the control scramble line T0 in all experiments, but no signal was visible in the knock down T1 line. Two sample western blots are shown in Figure 5.4.

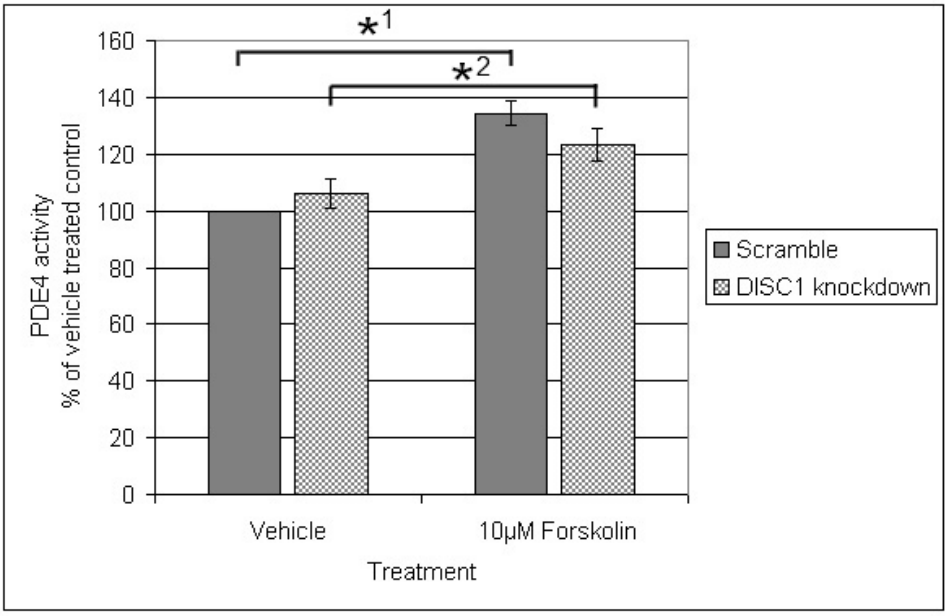


Figure 5.3A: Graph to show the effect of 10μM forskolin treatment on PDE4 activity in control and DISC1 knockdown SHSY5Y cells. A significant increase in PDE4 activity occurs in both cell lines on treatment with forskolin: *1, n=7, p=0.000003 (scramble), *2, p=0.04 (knockdown). The mean PDE4 activity of the vehicle treated control is 91.8pmoles/mg/min.

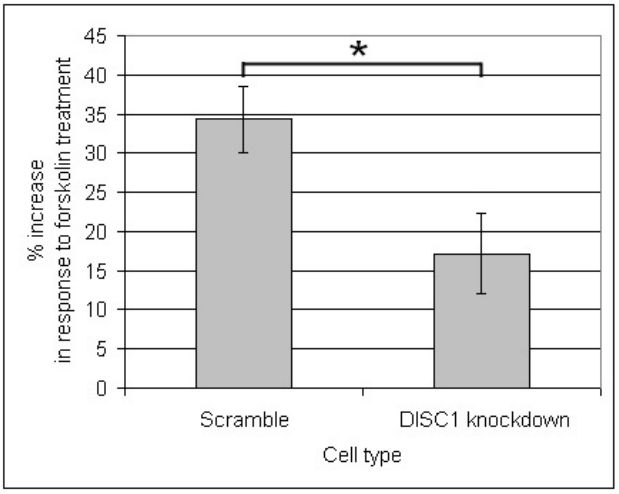


Figure 5.3B: Graph showing the percentage increase in PDE4 activity in response to treatment with 10μM forskolin. PDE4 activity increases significantly from unstimulated levels in both cell lines, but the increase in the knockdown cell line is significantly lower than that observed in control shRNA expressing cells (n=7, p=0.02).

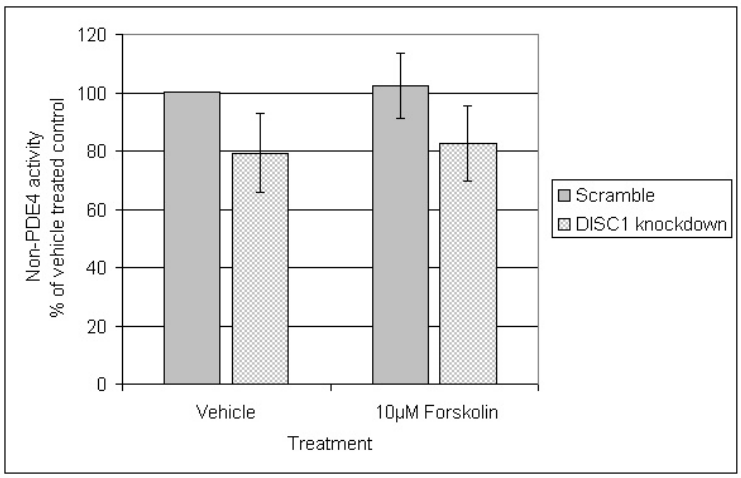


Figure 5.3C: Graph to show the effect of 10μM forskolin treatment on non-PDE4 activity in control and DISC1 knockdown SHSY5Y cells. There are no significant changes observed, suggesting the non-PDE4 response remains at baseline. The mean non-PDE4 activity of the vehicle treated control is 21.8pmoles/mg/min.

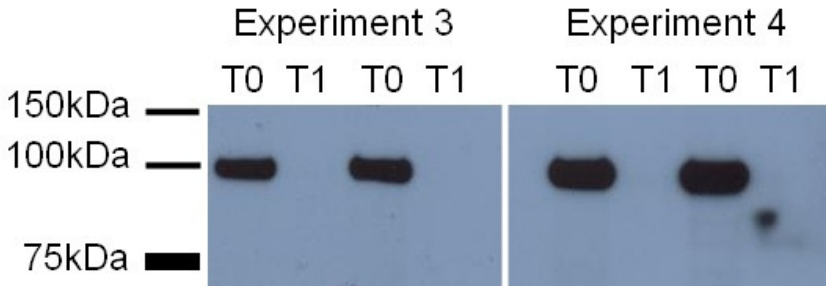


Figure 5.4: Sample Western blot from experiment 5.2.2. At 10 mins exposure a clear signal is seen at 100kDa using the αDISC1 antibody in the control scramble line (T0). No signal is visible from the knockdown cell line (T1).

5.2.3 DISC1 overexpression amplifies the response of PDE4 activity to short term lithium treatment

DISC1 overexpression was induced in TRTODISC1 by treatment for 24 hours with 1μg/ml tetracycline, with cells being serum starved for the final four hours of treatment. Cells were then treated for 30 mins with vehicle or LiCl at a final concentration of 10mM. Both uninduced and induced cells showed a significant decrease in PDE4 activity in response to treatment with lithium chloride (Figure 5.5A). In the cells overexpressing DISC1, the decrease in PDE4 activity in response to lithium was significantly greater than in uninduced cells (Figure 5.5B). In the uninduced cells, the non-PDE4 response to lithium was also statistically significant, but did not alter with regard to DISC1 expression status (Figure 5.5C).

Again, western blots were performed to ensure DISC1 was induced where expected. Sample blots from 2 of the 3 experiments are shown in Figure 5.6.

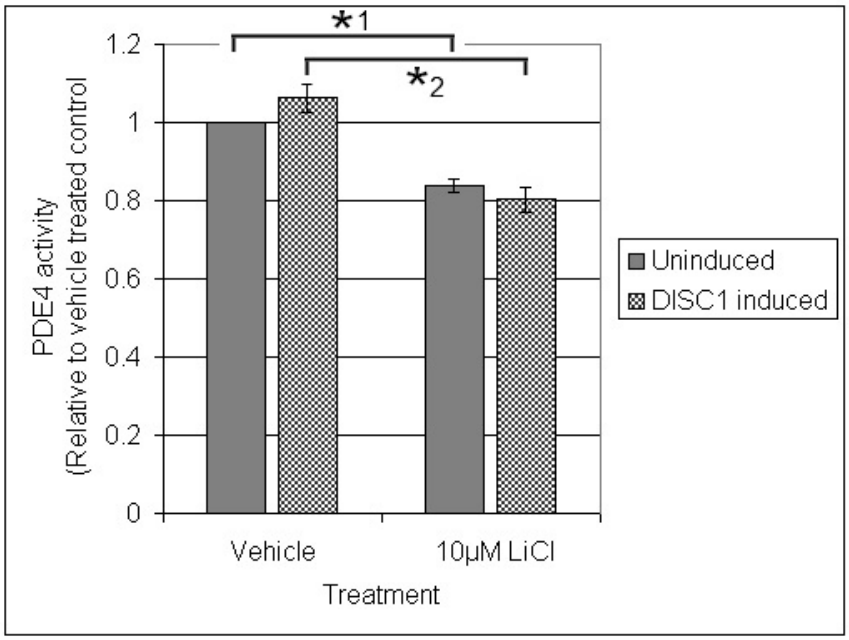


Figure 5.5A: Graph showing the effect of 10mM lithium treatment on PDE4 activity in DISC1 inducible SHSY5Y cells. The effect of lithium treatment is significant in cells regardless of the DISC1 status: *1, n=3, p=0.0008 (uninduced), *2, p=0.007 (DISC1 induced). Mean PDE4 activity of the vehicle treated control is 110.1pmoles/mg/min.

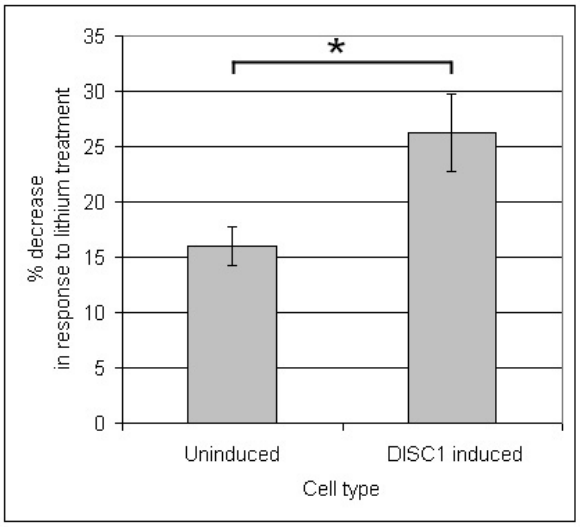


Figure 5.5B: Graph showing the percentage decrease in PDE4 activity in response to treatment with 10mM lithium. The decrease in cells overexpressing DISC1 is significantly larger than that observed in uninduced cells: n=3, p=0.05.

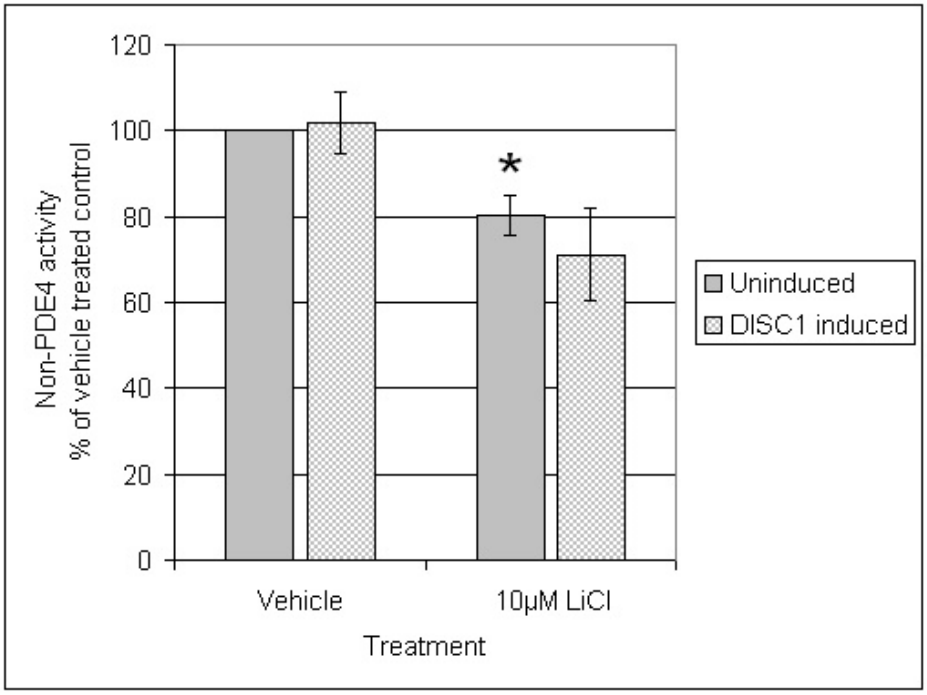


Figure 5.5B: Graph showing the effect of 10mM lithium treatment on non-PDE4 activity in DISC1 inducible SHSY5Y cells. The effect of lithium treatment is significant in uninduced cells: n=3, p=0.01. The mean non-PDE4 activity of the vehicle treated control is 47.4pmoles/mg/min.

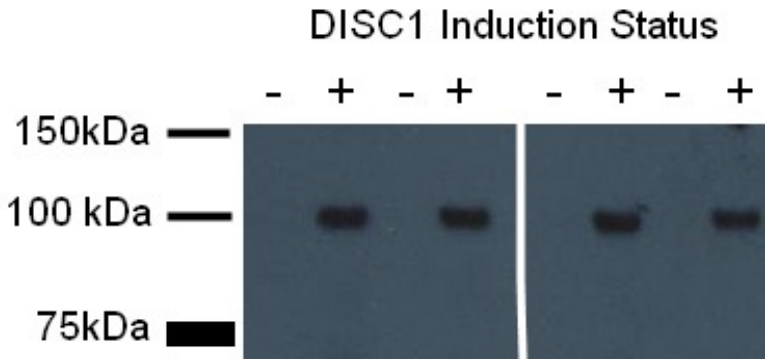


Figure 5.6: Sample western blot of two experiments from 5.2.3. At a short exposure time of 30 seconds, over expression of 100kDa DISC1 is clearly visible in induced cells.

5.2.4 Short term DISC1 overexpression has the same effect as LiCl on the PDE4 activity increase induced by forskolin

Thus far I have shown that both DISC1 overexpression and knock down has significant effects in modulating the increase in PDE4 activity in response to a forskolin challenge, which is presumed from the literature to be a result of PKA activation (MacKenzie et al., 2002). I have also shown that LiCl decreases basal PDE4 activity, and that overexpression of DISC1 augments this effect. Thus, as the final experiment in this investigation, DISC1 was overexpressed in combination with forskolin alone, and forskolin treatment plus lithium, to try and discern whether DISC1 and PDE4 were acting in the same signalling pathways.

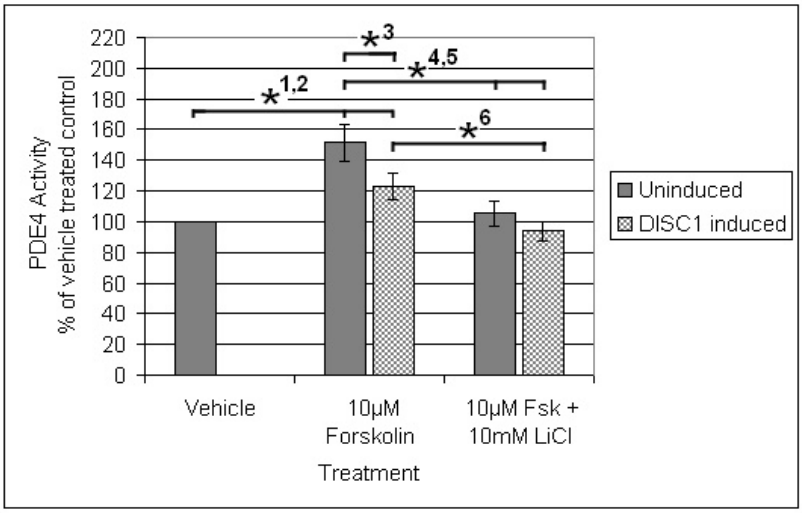
As before, DISC1 overexpression was induced by 24 hours of treatment with 1μg/ml tetracycline, and for the final 4 hours cells were serum starved. Cells were treated with vehicle, 10μM forskolin alone, or 10μM forskolin plus 10mM LiCl, lysed and PDE assayed as previously described.

As expected, after 9 experiments, treatment of the uninduced cells with forskolin alone produced a highly significant increase ($p=0.0001$) in PDE4 activity. Cells with induced DISC1 this time showed a small increase in activity in response to forskolin, but this increase was still significantly lower than the increase due to forskolin alone ($p=0.02$). LiCl treatment had a similar effect to overexpression of DISC1, in this case completely preventing the forskolin stimulated increase in PDE4 activity. There was no additive effect of both overexpressing DISC1 and treating with LiCl. Thus, it appears that DISC1 overexpression and lithium treatment have the same effect on forskolin stimulated increases in PDE4 activity. Both treatments lead to PDE4 activity remaining at baseline when cells undergo a forskolin challenge (Figure 5.7A).

As for the non-PDE4 component of the measurable activity, there was a significant effect of treatment with both lithium and forskolin, regardless of the DISC1 induction

status (Figure 5.7B). This may be because the stimulation of the cells alters the accessibility of other PDEs, for example to GSK3β.

As with the other experiments, western blots were performed to ensure DISC1 overexpression was as expected. Sample blots are shown in Figure 5.8.



Figures 5.7A: A graph showing the effect of 10μM forskolin treatment in combination with 10mM lithium treatment on PDE4 activity in DISC1 inducible SHSY5Y cells. There is no measurement taken from vehicle treated induced cells as the throughput of each experiment is limited to 5 samples. In previous experiments there was no significant DISC1 dependent difference in basal PDE4 activity (Figures 5.1A and S.2.2). Forskolin treatment results in an increase in PDE4 activity in uninduced and DISC1 induced cells: $*^1$ p=0.0001, $*^2$ p=0.01. Despite this, the increase in DISC1 induced cells is significantly less than in uninduced cells: $*^3$ p=0.02. Addition of LiCl prevents the PDE4 activity increase in response to forskolin: $*^4$ p=0.006, $*^5$ p=0.0004. Finally, PDE4 activity in cells with DISC1 induction and lithium treatment is significantly lower than cells with DISC1 induction alone: $*^6$ p=0.01. There is no difference in PDE4 activity in lithium treated cells, regardless of DISC1 induction status, suggesting there is no additive effect of both treatment. The mean PDE4 activity in the vehicle treated control is 101.3pmoles/mg/min.

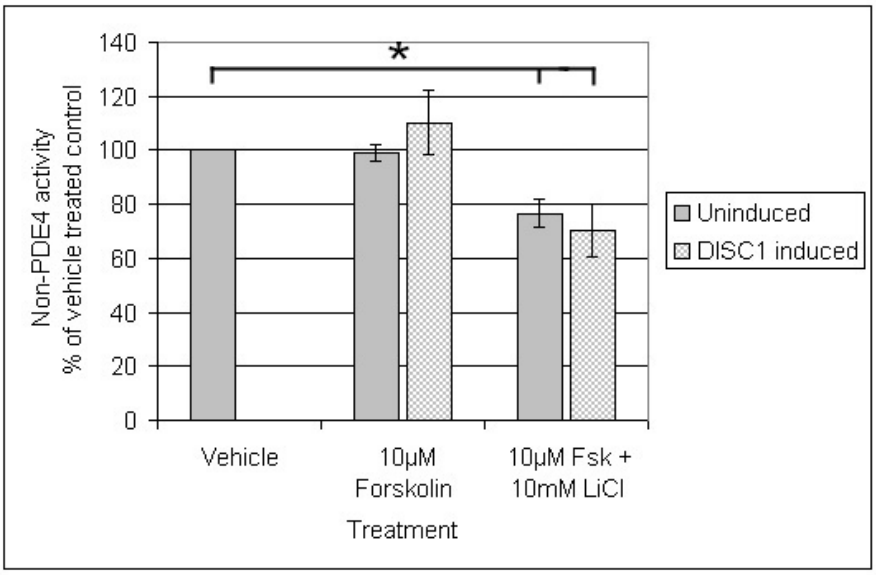


Figure 5.7B: Graph showing the effect of 10μM forskolin treatment in combination with 10mM lithium treatment on non-PDE4 activity in DISC1 inducible SHSY5Y cells. The combination of forskolin and lithium treatment is significant regardless of DISC1 induction status:* n=9, p=0.0003 (uninduced), p=0.008 (DISC1 induced). The mean non-PDE4 activity of the vehicle treated control is 27.8pmoles/mg/min.

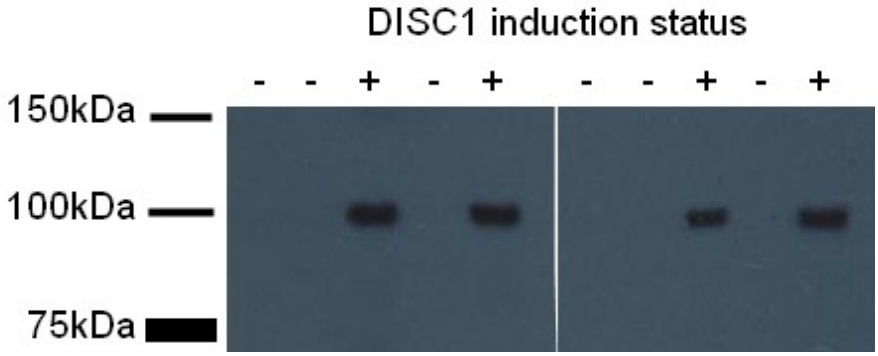


Figure 5.8: Western blot confirming overexpression of DISC1 in induced cells. A clear signal is evident at 100kDa after only 30 seconds exposure using the αDISC1 antibody.

5.2.5 DISC1 knockdown does not affect the response to short term lithium treatment

DISC1 knock down (T1) cells and control scramble shRNA cells (T0) were serum starved overnight before being treated with vehicle or 10mM LiCl. Cells were lysed and PDE activity assayed as previously described. Treatment of both cell lines with LiCl resulted in a significant decrease of PDE4 activity from basal activity levels, which was within the ranges observed in previous experiments (Figure 5.9A, Figures 4.3A, 4.4A). There was no DISC1 dependant difference in the magnitude of the decrease. There were no significant changes observed in the non-PDE4 component of the measurable activity (Figure 5.9C). As previously, western blots were performed to ensure DISC1 was knocked down as expected. This was the case, and two sample blots from this experiment are shown in Figure 5.11.

During this experiment it was also observed that as the knock down cells reached a higher passage number, the basal PDE4 activity in these cells decreased (Figure 5.10). This decrease in PDE4 activity increased as cell passage number increased over P15, and was not observed in experiments performed with cells at a lower passage number (Figure 5.3A). This meant that in this experiment, basal PDE4 activity was significantly lower in DISC1 knock down cells than in cells expressing a control scramble shRNA at the same passage number ($n=6$, $p=0.01$).

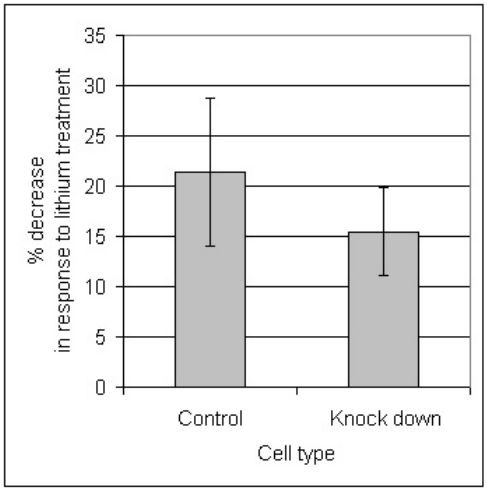


Figure 5.9A: Graph showing the decrease in PDE4 activity in response to treatment with 10mM lithium in control scramble and DISC1 knockdown cells. Both cell lines produce a decrease in activity which is significantly different to basal activity levels for that line (n=6, p=0.02 for both cell lines), but there is no difference between them in magnitude of the response.

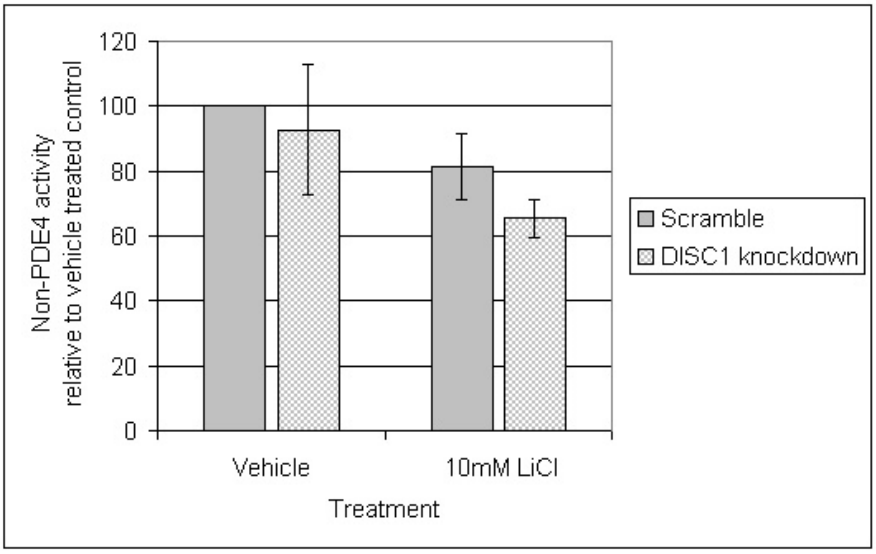


Figure 5.9B: Graph to show the effect of 10mM lithium treatment on non-PDE4 activity in control and DISC1 knockdown SHSY5Y cells. No significant changes in activity are observed. n=6. Mean non-PDE4 activity of the vehicle treated control was 25.5pmoles/mg/min.

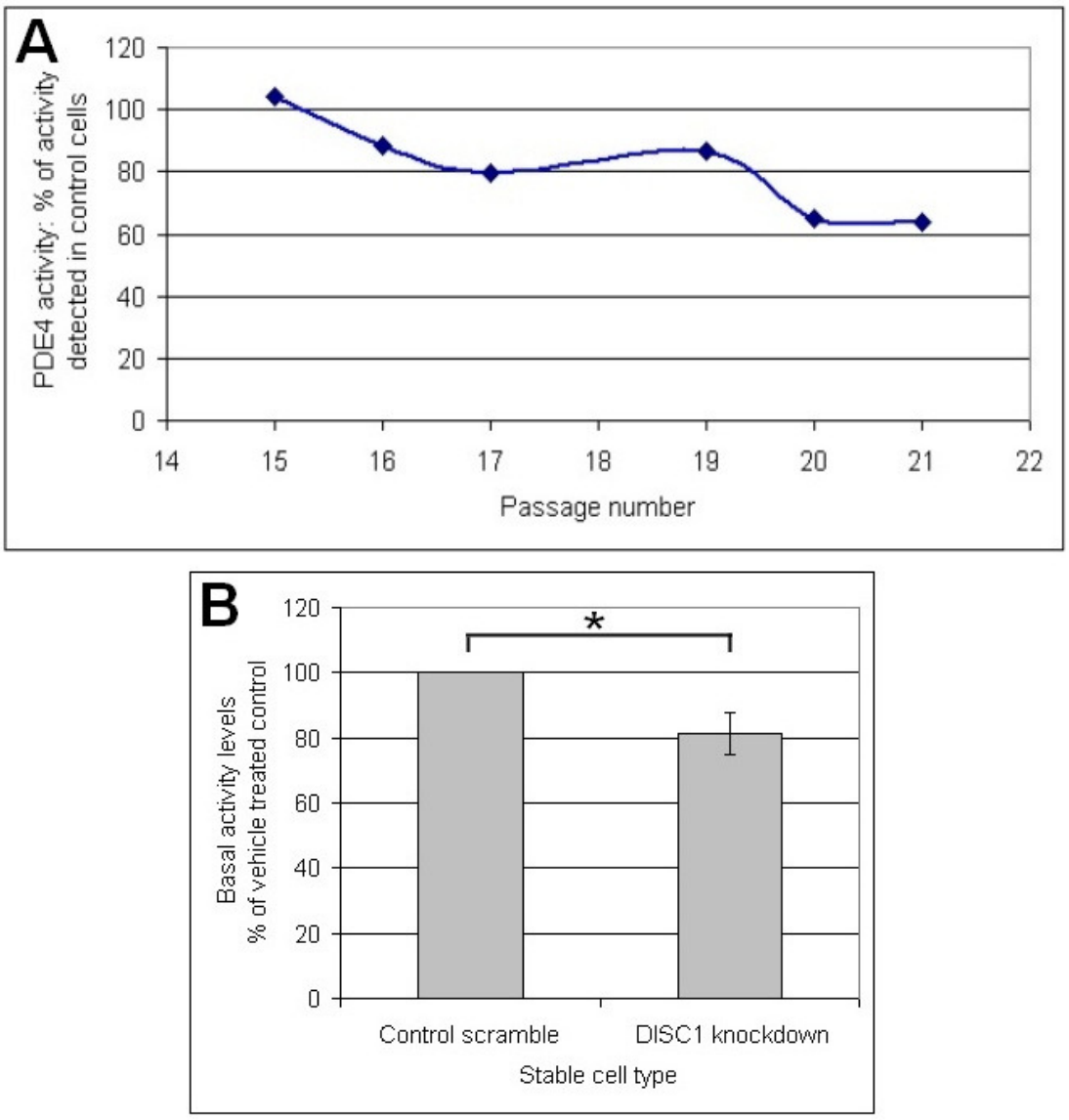


Figure 5.10A: Basal PDE4 activity decreases in DISC1 knockdown cells as higher passage numbers are reached. B: This results in DISC1 knockdown cells possessing significantly lower basal PDE4 activity than the control scramble cell population (n=6, p=0.01). Mean PDE4 activity of the control scramble line was 114.3pmoles/mg/min.

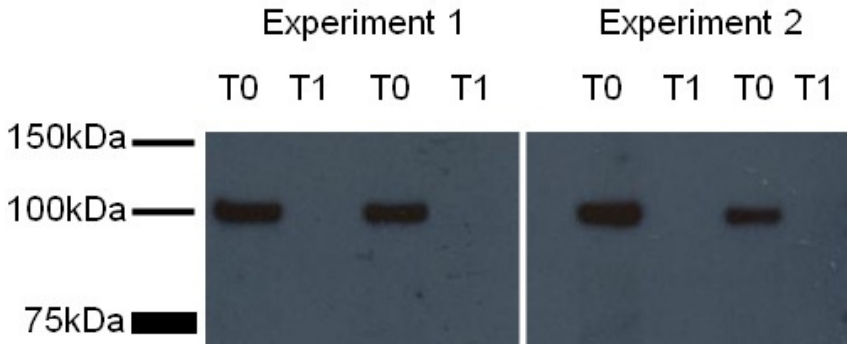


Figure 5.11: Sample Western blot from two replicates during experiment 5.2.4. At 10 mins exposure a clear signal is seen at 100kDa using the αDISC1 antibody in the control scramble line (T0). No signal is visible from the knockdown cell line (T1).

5.2.6 Changes in PDE4 activity are not due to changes in PDE4B or PDE4D protein expression

As has already been demonstrated in 3.3.7.3, there is no change in PDE4A, C or D mRNA expression in either of the stable cell lines. To analyse the expression of protein in these cell lines, 4 western blots from each of the cell lines were probed with the pan PDE4B and pan PDE4D antibodies, along with a GAPDH loading control. The αDISC1 antibody was kindly supplied by Professor Akiyama, University of Tokyo, while panPDE4B and panPDE4D were kind gifts from Miles Houslay at the University of Glasgow. The strength of signal from each antibody was analysed using densitometry on ImageJ software, then normalised to GAPDH signal for that lane and compared.

Staining of these blots with the pan PDE4B antibody produced a doublet of PDE4B1 and PDE4B3 at approximately 82kDa (Millar et al., 2005b). Densitometry was performed on this doublet. Staining with the pan PDE4D antibody produced a series of bands (there are at least 12 recorded human isoforms of PDE4D (http://www.ebi.ac.uk/uniprot)), and the bands that densitometry were performed on were those that migrated to approximately 98kDa, 86kDa and 80kDa. It is difficult to

predict precisely which isoforms these are, as there are so many similarly sized PDE4D isoforms, but PDE4D3, 4, 5, 7, 8 and 9 all migrate to this area of a gel (Richter et al., 2005). There were no gross differences in PDE4B or PDE4D protein expression, regardless of the DISC1 status of the stable cell lines (Figure 5.12A-D). This is consistent with the results obtained from the investigation into mRNA levels.

A

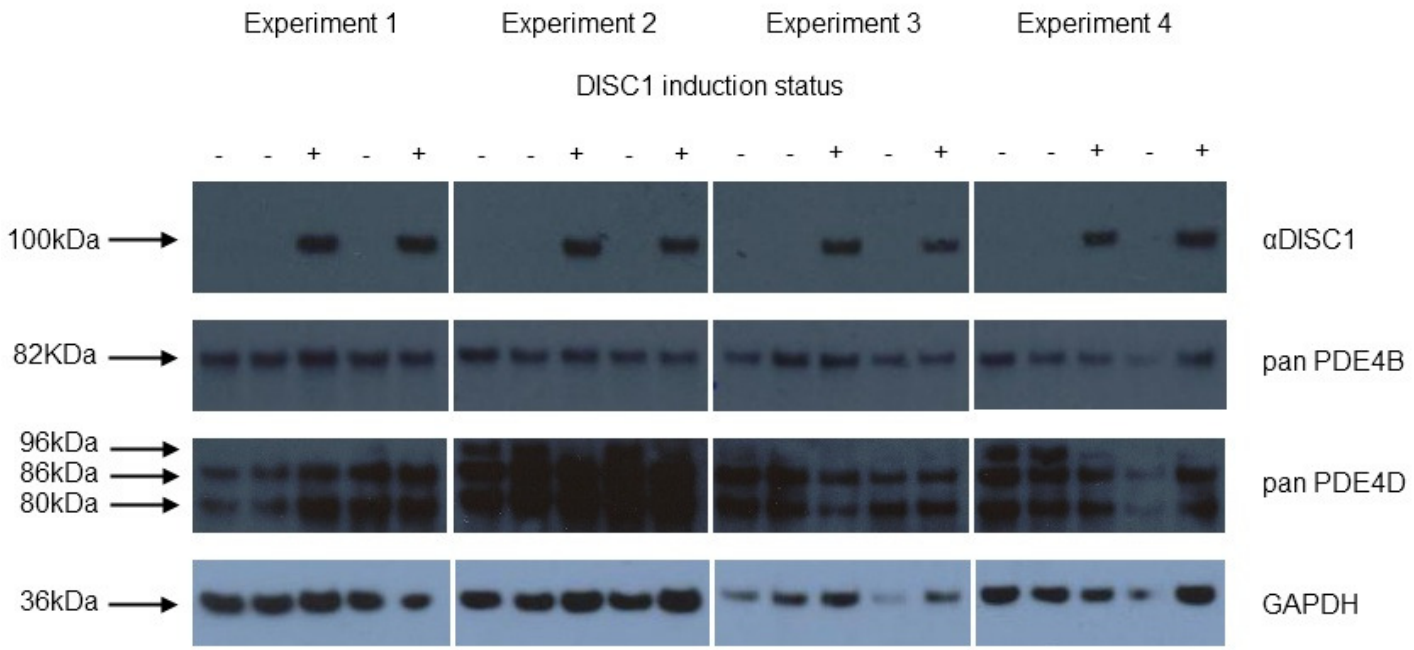


Figure 5.12A: Western blots probed with αDISC1, pan PDE4B, pan PDE4D and GAPDH antibodies to compare the expression of protein with regards to the DISC1 induction status of TRTODISC1. Clear induction of DISC1 can be seen after 30 seconds of exposure. At this exposure time, the signal from endogenous DISC1 is not visible. No gross differences can be observed in the expression of PDE4B or PDE4D protein with respect to DISC1 overexpression, once the signal is normalised to the GAPDH loading control.

B

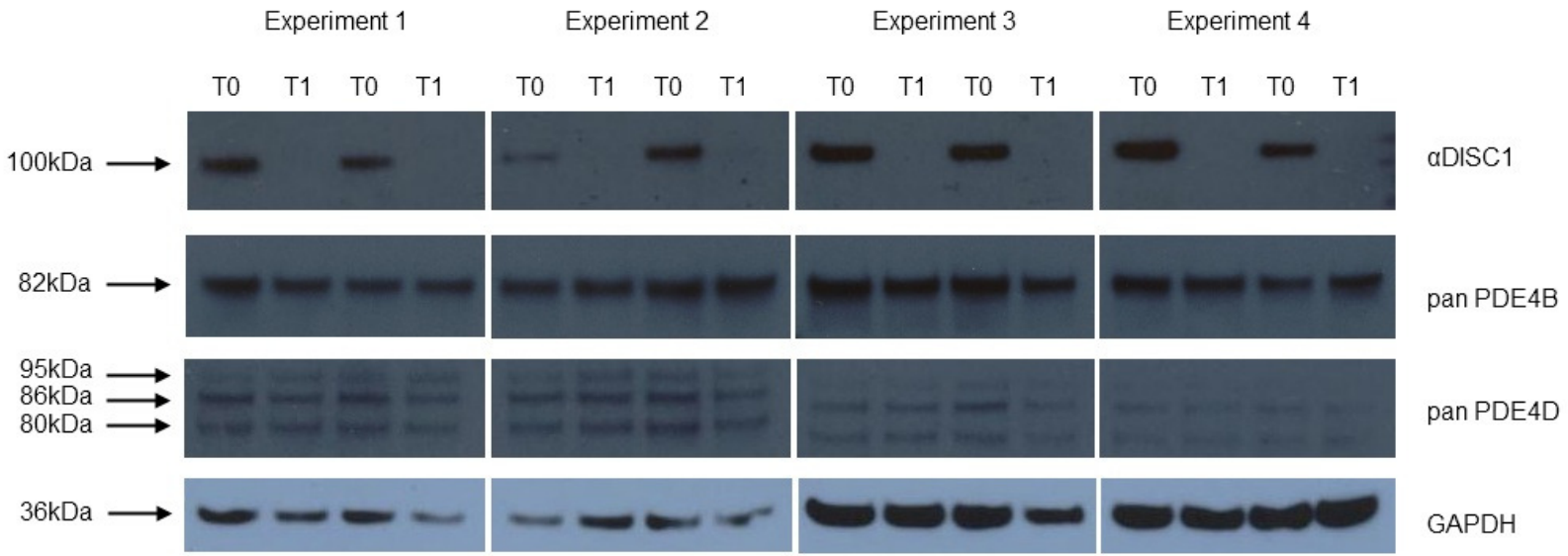


Figure 5.12B: Western blots probed with αDISC1, pan PDE4B, pan PDE4D and GAPDH antibodies to compare the expression of protein in DISC1 knockdown (T1) cells versus cells expressing the scramble control shRNA (T0). A clear DISC1 signal can be seen in the control cells after 5 minutes of exposure time. No gross difference can be observed in the expression of PDE4B or PDE4D protein with respect to DISC1 presence, once the signal is normalised to the GAPDH loading control.

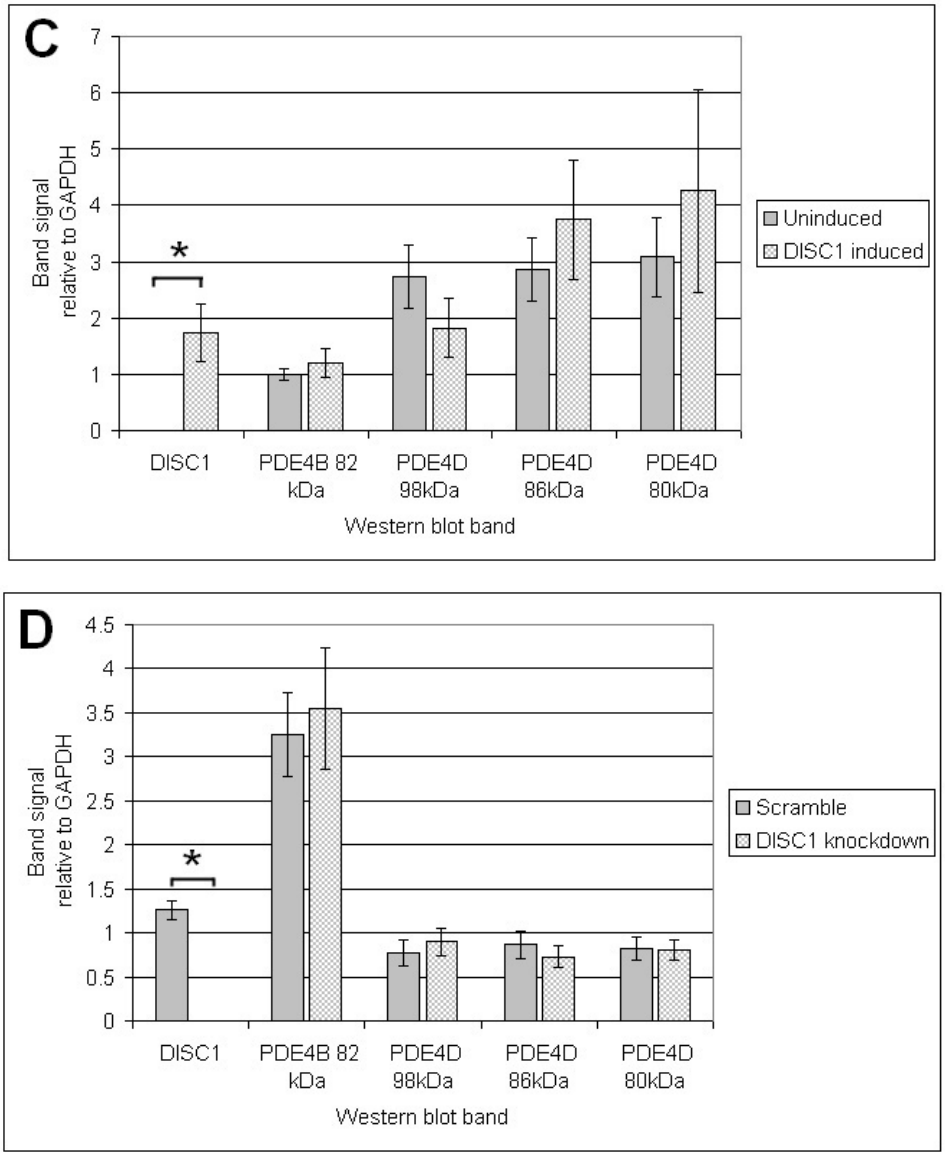


Figure 5.12: Graphs showing densitometry results from DISC1 overexpressing and knockdown cells. The only significant differences observed are from DISC1. C: In the overexpressing line, a 100kDa DISC1 signal is clear after 30 seconds exposure, while no endogenous signal is apparent. D: After 5 minutes exposure, DISC1 in control cells is clearly visible, with no signal from the knockdown cells. $n=8$, $p<0.0001$. There is no differential expression of PDE4s in either cell line.

5.2.7 DISC1 overexpression and knock down has no effect on the localisation of exogenous PDE4B and GSK3β

As was seen in 4.4.5, transiently transfected PDE4B-Flag and GSK3β-myc colocalise throughout the cytoplasm of SHSY5Y cells. After noting effects of DISC1 on the activity of PDE4s, I performed the same immunocytochemistry on the DISC1 overexpressing cell line, and on the DISC1 knockdown cell lines. Colocalisation of PDE4B-flag and GSK3β-myc was analysed using the Intensity Correlation Analysis (ICA) plugin for ImageJ (Li et al., 2004, section 2.3.5.2), and the Pearson Correlation co-efficient, Mander's overlap coefficient and Intensity Correlation Quotient (ICQ) obtained for comparison.

All images showed clear colocalisation of PDE4B-flag and GSK3β-myc, with Pearson correlation coefficients ranging between 0.429 and 0.752 (where -1 represents exclusion, 0 random localisation, and 1 complete correlation), Mander's coefficients ranging between 0.745 to 0.905 (where 0 represents low and 1 represents high colocalisation) and ICQs ranging between 0.14 and 0.282 (where 0 equals random staining, 0>x>-0.5 shows segregated staining, and 0.5>x>0 dependent staining). In addition, intensity correlation plots for both PDE4B-flag and GSK3β-myc were positively skewed, indicating dependant staining (Figure 5.13A-C) (Grünewald et al., 2009).

3 images of each cell type (TRTODISC1 with and without DISC1 induction, T0 scramble control and T1 DISC1 knockdown) were analysed using the ICA plugin. The above coefficients were compared, and no difference in colocalisation was observed when DISC1 status changed (Figure 5.14A-F, 5.15A-F). Correlation coefficients remained stable regardless of the levels of DISC1 present (Figure 5.16A-C). Overexpressing or knocking down DISC1 therefore has no effect on the localisation of PDE4B-flag and GSK3β-myc. It would be interesting to look at the effect of DISC1 on endogenous protein if a suitable GSK3β specific antibody could be obtained.

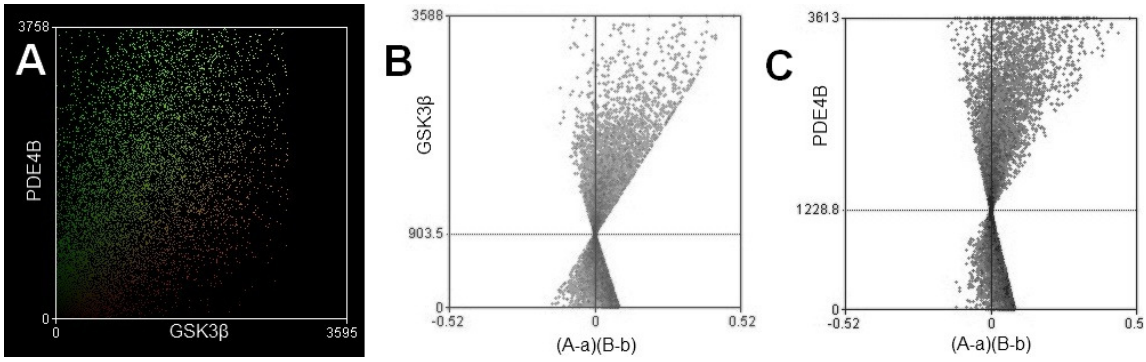


Figure 5.13: A is a correlation scatter plot depicting red versus green intensity in image 5.14C. In this plot yellow pixels denotes colocalisation. B and C show intensity correlation plots of GSK3β and PDE4B staining respectively. These graphs plot the differences from the mean intensity of each channel for each pixel, and a positive skew of both graphs indicates dependent staining. This same formula is used to form the ICQ values.

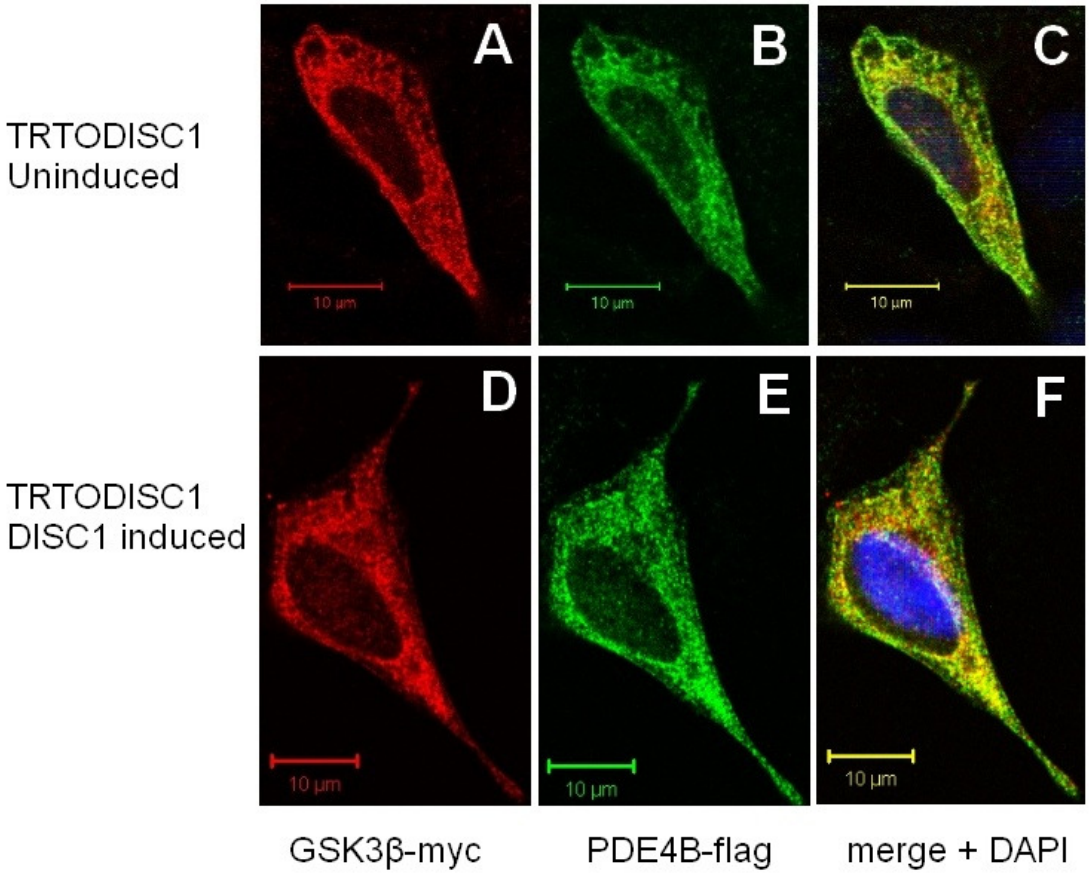


Figure 5.14 (previous page): Immunocytochemistry showing staining of transiently transfected exogenous GSK3β-myc and PDE4B-flag in control cells (A-C) and cells with DISC1 overexpression induced for 24 hours (D-F). Induction of DISC1 does not result in any gross differences in localisation of these exogenous proteins.

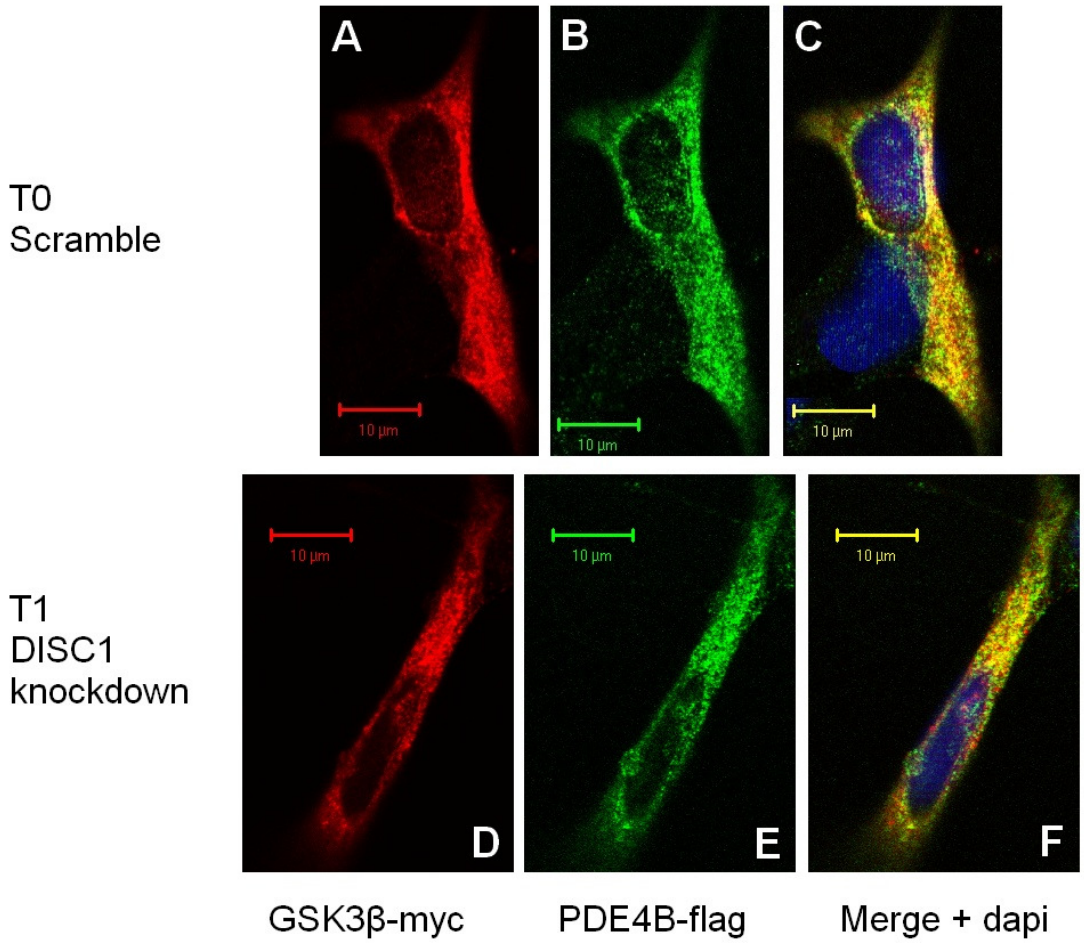


Figure 5.15: Immunocytochemistry showing staining of transiently transfected endogenous GSK3β-myc and PDE4B-flag in control scramble shRNA cells (A-C) and DISC1 knockdown cells (D-F). Knockdown of DISC1 does not result in any gross differences in localisation of these endogenous proteins.

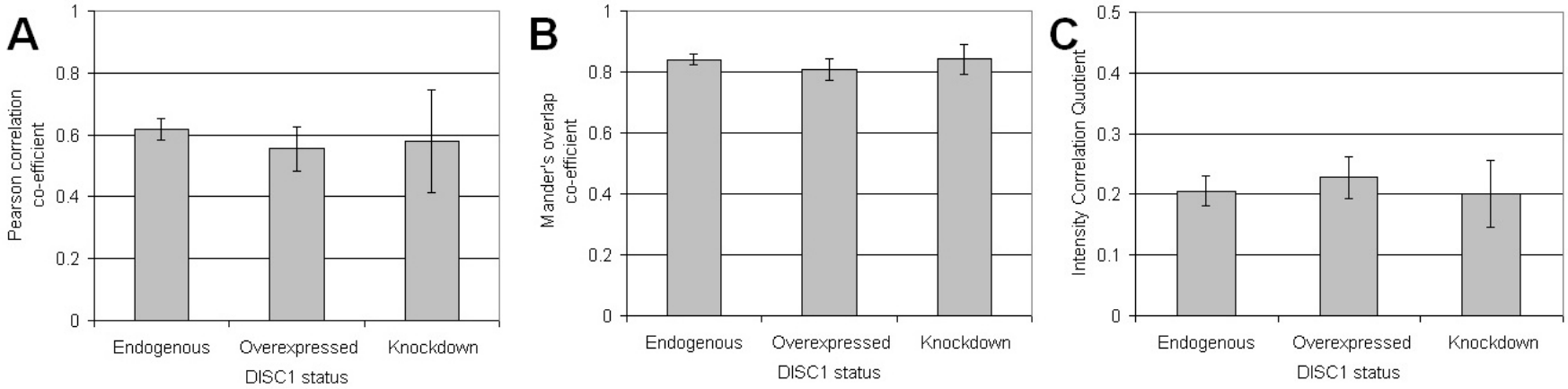


Figure 5.16: A shows the Pearson correlation co-efficient of GSK3β-myc and PDE4B-flag remains stable despite changes in DISC1 expression, where 0 equals random localisation and 1 absolute colocalisation. B shows the Mander's overlap coefficient also remains stable regardless of changes to DISC1 expression. Here 0 is low colocalisation and 1 high colocalisation. Finally, C shows that the intensity correlation quotient also remains stable, where a negative value equals segregated staining, and a positive value between 0 and 0.5 demonstrates dependent staining.

5.3 Discussion

In this chapter I have shown that DISC1 may be responsible for some of the fine control of PDE4 activity by GSK3β, and potentially also by PKA. DISC1 is not, however, a necessary and essential modulator of PDE4 activity, as both forskolin and lithium treatment affect PDE4 activity when DISC1 is knocked down. Changes in DISC1 do alter the magnitude of the response to these drugs, suggesting that the presence of DISC1, most likely as part of a multi-component and dynamic signalling complex, can help to fine tune the responses to these different modulators.

5.3.1 DISC1, GSK3β and Lithium

We saw in the previous chapter that inhibition of GSK3β by lithium and the specific inhibitor SB216763 results in cAMP accumulation, and a highly significant and replicable decrease in basal PDE4 activity ($p<0.01$). This suggests that GSK3β, be it directly or indirectly, has a tonic activating effect on basal PDE4 activity. Mao *et al* (2009) demonstrated that DISC1 has the capacity to inhibit GSK3β, but this was not initially apparent with regards to GSK3β-mediated effects on PDE4 activity in this experiment, where overexpression of DISC1 did not result in decreased basal PDE4 activity.

However, DISC1 overexpression does potentiate the decrease in basal PDE4 activity that occurs in response to LiCl treatment. PDE4 activity of control uninduced cells decreased by 16% (+/-1.7) on lithium treatment, in DISC1 induced cells the decrease was 26% (+/-3.5). This finding challenges the current literature. If there is inhibition of GSK3β by DISC1 under basal conditions, this effect is somehow compensated for, with respect to PDE4 activity. Other proteins in the DISC1 signalling complex may maintain GSK3β-mediated modulation of PDE4 activity, despite overexpression of DISC1, perhaps by blocking the inhibitory effect of DISC1 upon GSK3β activity. When the

activity of GSK3β is altered, by lithium treatment, the cellular context may change so that DISC1 inhibition of GSK3β takes effect, decreasing PDE4 activity yet further.

An alternative hypothesis, contrary to the inhibitory interaction reported by experiments using fragments of DISC1 to bind GSK3β by Mao *et al*, 2009, can be formed on the basis that DISC1 does not inhibit GSK3β under basal conditions. There are then two ways that overexpressed DISC1 may act to augment the effect of lithium on PDE4 activity. Firstly, the presence of excess DISC1 may sensitise the cells to lithium, by altering the binding partners present in a multi-component complex. For example, DISC1 has shown activating effects on AKT in some cell types (Hashimoto et al., 2006), and AKT is known to phosphorylate and inactivate GSK3β (Cross et al., 1995). Secondly, overexpression of DISC1 may force an inhibitory interaction, such as that reported by Mao *et al*, between full length DISC1 and GSK3β that does not occur at normal levels of DISC1 expression. Figure 5.17 is an attempt to illustrate these concepts.

In contrast, DISC1 knockdown had no effect of the magnitude of the PDE4 response to lithium, which could be predicted if DISC1 was only acting as a GSK3β inhibitor under certain cellular contexts, such as increased cAMP, or overexpressed DISC1.

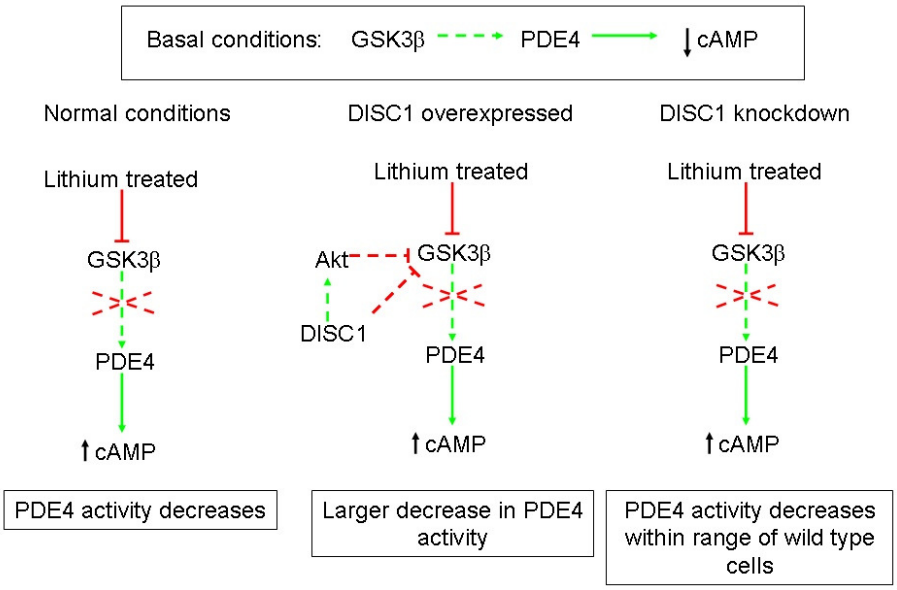


Figure 5.17: Schema depicting the effect of changes in DISC1 status on the response of PDE4 activity to lithium. Solid green arrows indicate activation events reported in the literature, dashed green arrows represent possible interpretations of my experimental results. Red dotted lines represent the different hypotheses detailed in the main text as to why presence of DISC1 is affecting the magnitude of changes in PDE4 activity. Under basal conditions, GSK3β tonically activates PDE4, leading to a decrease in local cAMP. Thus, when lithium is added, PDE4 activity decreases and cAMP concentration increases. Overexpression of DISC1 plus lithium treatment results in a larger decrease in PDE4 activity, potentially due to increased GSK3β inhibition by DISC1, while DISC1 knockdown has no effect on the lithium related decrease in PDE4 activity. In this paradigm, DISC1 may be inhibiting GSK3β directly, or indirectly, through increased AKT activation for example.

5.3.2 DISC1, GSK3β and the induction of PDE4 activity in response to forskolin

The data presented here demonstrate that both short term induction of DISC1 and treatment with lithium suppressed the increase in PDE4 activity in response to a forskolin challenge. In the first of these experiments, DISC1 overexpression prevented PDE4 activity increasing from baseline. Lithium treatment had the same effect. In the second DISC1 overexpression experiment, PDE4 activity did not remain at baseline, but increased by a significantly smaller amount then in uninduced cells: a 23% (+/-8) increase compared to a 51% increase (+/-12). There was no additive effect on PDE activity changes when DISC1 overexpression and lithium treatment were combined.

These results suggest that in addition to PKA activity, GSK3β activity is also required for the forskolin stimulated increase in PDE4 activity to occur. Treatment of SHSY5Y cells with forskolin leads to activation of adenylyl cyclase, increased production of cAMP, and activation of PDE4. This activity increase is attributed to PKA phosphorylation, due to it being blocked by either PKA inhibitors or site directed mutation of the PKA phosphorylation site on PDE4 (depicted in Figures 4.6A & B MacKenzie et al., 2002). These results do not necessarily conflict with the evidence I have provided here. Lithium may simply be blocking PDE4 activity, so that no response to forskolin is possible. Alternatively, GSK3β may be acting downstream of, or synergistically with PKA, to produce the forskolin mediated increase in PDE4 activity. All activity assays performed by Mackenzie *et al* (2002) were performed in cell lysates, where GSK3β would have been active. PDE4B and PDE4D isoforms both have clusters of potential GSK3β phosphorylation sites (Ser145, Ser 150, Ser154 in PDE4B1 and Ser 138, Ser143, Ser 147 in PDE4D5) close to the PKA phosphorylation site (Ser133 in PDE4B1, Ser126 in PDE4D5). Although the spacing between these residues is non-canonical for GSK3β phosphorylation, this arrangement is conserved between long isoforms of PDE4B and PDE4D (Figures 4.6AandB).

If this model is correct, then there are in fact two possible mechanisms through which DISC1 may act to inhibit a forskolin mediated increase in PDE4 activity. Firstly, DISC1 may directly inhibit GSK3β, as suggested by Mao *et al*, but only under conditions of increased cAMP. Secondly, DISC1 may prevent phosphorylation of PDE4 by PKA. Both these mechanisms would result in suppression of the forskolin stimulated increase in PDE4 activity. I have attempted to illustrate this model in Figure 5.18.

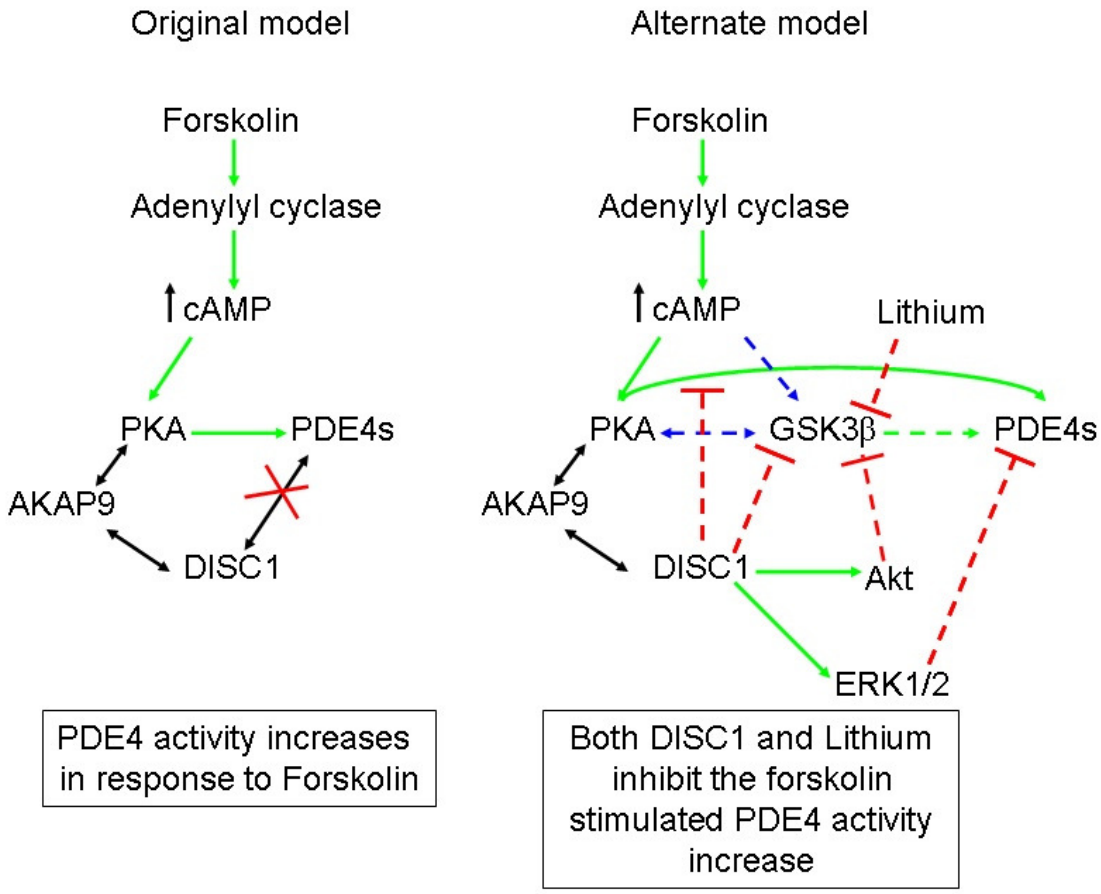


Figure 5.18: A schema to illustrate the alternate model proposed in the main text by which overexpressed DISC1 and lithium act to inhibit the forskolin stimulated increase in PDE4 activity. Activating events reported in the literature are depicted as solid green arrows. In the original model, forskolin challenge results in activation of adenylyl cyclase, increased cAMP and activation of PKA. PKA then phosphorylates PDE4s to increase their rate of activity and causing dissociation of some PDE4 isoforms from DISC1. All these components have

been shown to interact with AKAP9, a scaffolding protein involved in assembly of signalling complexes (Conti et al., 2003a, Camargo, 2006, Millar et al., 2003, Wong and Scott, 2004). In the alternate model, GSK3β activity is required for PDE4 activation to occur. Thus, inhibition of GSK3β activity by lithium results in no change of PDE4 activity in response to forskolin. There are multiple mechanisms by which DISC1 can have the same effect, these are detailed in the main text and illustrated by dashed red lines. Depicted in blue are interactions reported in the literature that may conflict with this model. A discussion of these points follows in the main text.

There are numerous potential mechanisms for how overexpressed DISC1 may interrupt phosphorylation of PDE4 by PKA. Firstly, excess DISC1 may bind to PDE4 and prevent access to the phosphorylation site by PKA. In both PDE4B and PDE4D, the PKA phosphorylation site is relatively close to the common DISC1 binding site, 78 amino acids away in PDE4B and 75 amino acids in PDE4D (see Figures 4.6A and B). Recent work solving the crystal structure of PDE4D when bound to inhibitors suggests that many of these compounds function by binding to the UCR2 domain of PDE4D, effectively "closing" the active site by enhancing interactions between UCR2 and the active site. DISC1 interacts with both the UCR2 and catalytic domains of PDE4D, implying that it may function in the same mechanism (Burgin et al., 2010). This work does not offer an explanation as to how some PDE4 isoforms are released from DISC1 in response to forskolin stimulation. In conditions where 100kDa DISC1 is overexpressed, some PDE4 isoforms, including PDE4A5 and PDE4B1 would not be released on forskolin treatment (Murdoch et al., 2007), potentially preventing an increase in PDE4 activity.

A further potential explanation is that the excess DISC1 simply sequesters PDE4 away from its sites of activation. Immunocytochemistry revealed an increased presence of DISC1, PDE4B and PDE4D in the nucleus when DISC1 was overexpressed (Figures 3.7 and 3.8), which may add some weight to this hypothesis. Finally, work by Hashimoto *et*

al (2006) raises the possibility that DISC1 may also be acting more indirectly, for example through ERK signalling. Overexpression of DISC1 in cortical neuron cultures results in increased levels of activated pERK1/2, with knockdown of DISC1 having the opposite effect (Hashimoto et al., 2006). Activated ERK2 phosphorylates PDE4s at their C-termini, within the catalytic domain. In long isoforms, this phosphorylation is inhibitory (Baillie et al., 2000), potentially because this phosphorylation stabilises the "closed" state of the PDE4 active site (Burgin et al.). DISC1 induction could therefore result in increased ERK activation, causing increased inhibitory phosphorylation of the long forms of PDE4. This pathway would have to be active only when cells are forskolin challenged, and not at basal levels of activity.

5.3.3 Evidence that may counter the alternate model

Long forms of PDE4s are already known to be activated by PKA. In this conclusion I have proposed an alternative model where GSK3β is also able to modulate PDE4 activity. I have presented a series of potential means by which this may be achieved. There is some evidence however, that may counter this model. DISC1 knockdown also had an effect on the forskolin stimulated increase in PDE4 activity. Knockdown of DISC1, perhaps contrary to expectations from the overexpression experiment, also resulted in a decreased magnitude of response to forskolin: a 17% (+/-5) increase compared to a 34% (+/-4) increase in control cells. If DISC1 were acting solely as an inhibitor of GSK3β, then knockdown would result in change to the magnitude of the PDE4 increase. This experiment emphasises the likely importance of all these changes occurring as part of a complex. While DISC1 might not be critical for the PKA mediated PDE4 increase to occur, it may bind another protein in the complex that is required for the elevation to occur to the expected magnitude. For example, it may bring GSK3β into close proximity with PKA and PDE4. As compartmentalisation is key to the activity of many of these proteins, it may simply be that certain PDE4 isoforms are not present in their usual locations if DISC1 is absent. This would have to occur on a very local scale, as no gross changes in PDE4B and PDE4D localisation were observed

by immunocytochemistry of DISC1 knockdown cell lines (Figures 3.9and3.10 A-F). Finally, there may be involvement of a third (or multiple) protein which interacts with DISC1 to mediate the response of PDE4 to PKA. Candidates for this third protein include AKAP9, one member of the AKAP family of proteins, which acts as a scaffold for assembly of a large array of components involved in signalling pathways, including PKA, PDE4, adenylyl cyclases and phosphatase enzymes (Wong and Scott, 2004, Conti et al., 2003b). AKAP9 was identified as a putative DISC1 interactor by yeast-2-hybrid screening (Camargo, 2006, Millar et al., 2003).

It is highly likely that even this more complex "alternate model" is still a simplified view of the actual events occurring in this experiment, and, by implication, in neurons. Indeed, there is evidence in the literature that conflicts with this hypothesis. PKA is known to inhibit GSK3β by phosphorylation at Ser9, in response to a forskolin challenge and G protein coupled adenylyl cyclase activity. The reduction in GSK3β activity was not complete however, reducing levels to 50% of control activity (Li et al., 2000). PKA reciprocally forms one of the targets of GSK3β, which phosphorylates the RII regulatory region of PKA (Brian et al., 1982). It is unclear from the literature what the purpose of this phosphorylation may be, but the leading theory is that phosphorylation of the RII region at this site can change localisation of catalytic subunits, as is seen with phosphorylation by cyclin B-p34cdc2 during the onset of mitosis. Phosphorylation decreases the affinity of PKA for AKAP9, and increases the affinity for AKAP95, resulting in a dynamic change in PKA localisation (reviewed Griffioen and Thevelein, 2002). Reciprocal phosphorylation of each of these proteins by the other could potentially switch activity on or off, or alter the localisation of critical pathway players, depending on the cellular context and presence of other proteins such as AKT and ERK. Thus the contradictory evidence from the literature that both GSK3β and PKA inhibit each other, yet from my work appear to be critical for response of PDE4 to forskolin, may simply suggest the presence of an autoregulatory cAMP responsive loop, that is not yet fully understood.

5.3.4 Adaptive changes observed in stable cell lines

The data presented here may also have shown the potential for the loss of DISC1 function to reset the normal activity levels of some of the involved signalling pathways. At high passage numbers the basal PDE4 activity of DISC1 knockdown cells began to decrease quite significantly (Figure 5.10), whereas activity levels in the scramble control cell line remained stable at the same passage numbers. Both cell lines were maintained on the same antibiotic, Zeocin, thus this was not a response to antibiotic treatment alone. This observation could imply a resetting of some of the cellular circuits involved with DISC1 function to a lower basal level of activity, to compensate for the loss of the modulating influence of DISC1.

In the majority of these experiments, there were no significant changes to the non-PDE4 component of measurable activity. In Figure 5.1B there is a small but significant increase in basal non-PDE4 activity after 24 hours of DISC1 induction, which may indicate the beginning of an adaptive response to DISC1 overexpression, but this was not seen in other experiments. In Figure 5.5C, treatment of uninduced cells with lithium causes a significant decrease in non-PDE4 activity: a trend towards this behaviour has been observed in other experiments. Finally, in Figure 5.7B, there is a significant effect of treating both induced and uninduced cells with forskolin and lithium combined, as the PDE4 activity drops significantly below basal activity. It is thus possible that while this system serves to maximise the ability to record changes in PDE4, the effect of lithium, and thus GSK3β, is not specific to PDE4s, but can also affect other PDEs. All these significant changes occur in the TRTODISC1 inducible cell line, where PDE4 activity occupies a slightly lower percentage of the measurable PDE activity (29% +/-5, n=10) as opposed to 12.5-20% observed in wild type SHSY5Y and T0/T1. This decrease appears to be due to a lower PDE4 activity, as total activity in vehicle treated controls recorded from the inducible cell line is decreased (10 random experiments, 29957dps +/-1137 in wild type SHSY5Y, 26400dps +/- 1291 in the inducible cell line, p=0.04). This is the only cell line that was maintained long term on Blasticidin (though antibiotics were

withdrawn for a week before treatment), thus an interaction with this drug may result in decreased PDE4 activity. I can find no evidence of this in the literature. Alternatively, as this cell line is clonal, it may be that a cell with lower endogenous PDE4 activity was selected for in the production of the inducible line.

5.3.5 PDE4 expression in stable cell lines

All of these observed changes in PDE4 activity occur on a background of stable PDE4 gene and (Figure 3.13A-D) protein expression (Figures 5.12A and B). This is consistent with observations from in house microarray studies on lymphoblastoid cell lines from the translocation family and DISC1 mutant mice that DISC1 mutations do not alter the expression of PDE4 isoforms (Brown *et al*, paper in preparation, Camargo *et al*, paper in preparation). Importantly this infers that the decrease in PDE4 activity observed at higher passage numbers in the knockdown line is not due to a change in protein expression, only enzyme activity.

Thus, as described earlier, it is difficult to reconcile all of my experimental results with the existing literature. The multi tiered regulation of enzymes such as PKA and GSK3β is so complex, that there may be existing feedback loops that we are as yet unaware of. What is clear is that while DISC1 is not essential for changes in PDE4 activity to occur, its presence can alter the magnitude of these changes, allowing for fine tuning of the PDE4 response. I have proposed a model for how DISC1 may be imposing effects on the SHSY5Y system employed here. In this model, activity of both GSK3β and PKA are required for PDE4 activity to increase in response to forskolin. The ability DISC1 possesses to bind a large number of other proteins may add to the contextual control of these enzymes, and the outcome of this control can be seen in PDE4 activity changes. PDE4 acts to break down cAMP, which will then feed back into these same signalling pathways. The exquisitely balanced control of such signalling makes it is easy to imagine how the loss or malfunction of a mediating protein such as DISC1, GSK3β or PDE4 may lead to dysregulation with time.

6 Using Primary Cortical Neurons as a model for measuring PDE4 activity downstream of NMDA and Dopamine Type I receptors

6.1 Introduction

In chapters 4 and 5, I described how SHSY5Y cells can be used successfully as a model for assessing PDE4 activity. SHSY5Y are ideal for such a purpose, as endogenous PDE activity is relatively high, and the majority of measurable activity is PDE4 specific (Figure 3.2A). SHSY5Y cells are derived from a human neuroblastoma, and as such have been used frequently in functional assays as models for a neural cellular environment (Millar et al., 2005b, Shinoda, 2007 #184), being easy to culture with a shorter lag time than using primary cultured neurons. They are not however, perfect models of the neural environment. SHSY5Y are a relatively stable cell line when undifferentiated, whereas neurons are highly differentiated large cells with multiple functionally specific compartments. SHSY5Y can be induced to develop certain neuronal features by retinoic acid induced differentiation (Encinas M et al., 2000). It is relatively common that a clear effect observed in cultured cell whole lysate, such as the effect of DISC1 knockdown on ERK1/2 phosphorylation in PC12 cells, stems from a localised sub cellular change in activity – the growth cone in this example (Shinoda et al., 2007).

Perhaps the largest difference between primary neuronal cultures and standard transformed cultured cells, is the large range of receptors expressed by primary neurons. The question of whether SHSY5Y cells express receptors that have been associated with schizophrenia pathophysiology such as dopamine type I (Iizuka et al., 2007, Machida et al., 2005, Nair V, 1996) and NMDA receptors (Jantas and Lason, 2009, Nair V, 1996,

Kulikov et al., 2007) is contentious. Furthermore, undifferentiated SHSY5Y cells do not form the dendritic structures where receptors are located in primary neurons. Primary cortical neurons clearly express dopamine type I receptors by 7 D.I.V, and expression continues until at least 14 D.I.V (Noh and Gwag, 1997, Nagai et al., 2007, Outeiro et al., 2009). There is also a consensus that functional NMDA receptors are expressed in primary neuron cultures within a similar timescale (Chen et al., 2005, Javier et al., 2007).

Agonism of these two receptors in particular was of interest both because of their potential role in schizophrenia (see the introduction for a basic review of the dopaminergic and glutamatergic theories), and because of their downstream signalling pathways. DRD1 is directly coupled to adenylyl cyclase to produce cAMP on receptor agonism, (Missale et al., 1998), and thus invokes PKA activation as seen by direct stimulation of adenylyl cyclase with forskolin. NMDA receptors are of interest for two reasons. Firstly, DISC1 interacts with AKAP9 (Mackie *et al,* manuscript in preparation,), the Yotiao fragment of which associates with the NR1 subunit of the NMDA receptor, and is found in complexes with NMDA receptors. Secondly, the calcium fluxes caused by agonism of NMDA receptors are closely coupled to cAMP fluxes: five of the isoforms of adenylyl cyclase are directly regulated by calcium (I, III, and VIII are stimulated, V and VI are inhibited). Indeed, adenylyl cyclase immunoreactivity is concentrated at hippocampal post synaptic densities in the striatum radium, thus positioned to respond to calcium fluxes initiated by NMDA receptor agonism and play a potent role in LTP (reviewed Cooper et al., 1995). Exposure of hippocampal brain slices to LTP inducing high frequency stimulation or a ten min NMDA bath results in an increase of cAMP concentration, which is blocked by NMDA antagonists (Chetkovich and Sweatt, 1993). cAMP concentration in hippocampal slices is increased at 1 and 5 minutes by an increase calmodulin dependant adenylyl cyclase activity in response to NMDA treatment (NMDA has an EC_{50} of 55μM for this effect), but returns to baseline by 20 minutes (Chetkovich and Sweatt, 1993).

It is clear that in SHSY5Y cells, where the effect of manipulations on PDE4 activity is easily detectable, that DISC1 and lithium are having comparable effects. To examine the wider significance of this model, and whether similar changes might be occurring downstream of schizophrenia associated receptors I prepared primary neuron cultures from CD1 and C57BL/6 background mice, and investigated their potential use as a model for assessing PDE4 activity. This assessment involved reverse transcription PCR to investigate which PDEs were expressed in these cultures, treatment of whole neuron lysates with PDE inhibitors to assess which PDEs were active, immunocytochemistry to look at localisation of receptors with respect to PDE4B and PDE4D, and treatment of cultures with A68930, a DRD1 specific agonist (DeNinno et al., 1991) and a combination of NMDA and Glutamate known to invoke significant calcium transients (Sanchez-Martin et al., 2006). I also wanted to test whether I could replicate the effect of the DISC1 point mutations described by Clapcote (Clapcote et al., 2007) on PDE4 activity in primary neuronal cultures using the standard PDE assay method used throughout the thesis. Finally, I attempted to replicate the effect of GSK3β inhibition on PDE4 that was observed in SHSY5Y cells in chapters 4 and 5.

6.2 Results

6.2.1 A wide range of cAMP PDEs are expressed in primary neuronal cultures

DNA sequences were obtained from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nuccore&itool=toolbar) for all major mouse cAMP PDE isoforms (Siuciak and Strick, 2006), and cross referenced to UCSC genome browser (http://genome.ucsc.edu/). Pan primers were designed for each of the following multiply spliced genes: PDE2A, PDE3A, PDE3B, PDE4A, PDE4B, PDE4C, PDE4D, PDE7A, PDE7B, PDE8A, PDE8B and PDE10A using the NCBI Primer Blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer pairs were tested on

cDNA prepared from whole C57BL/6 mouse brain, as there are reports in the literature of all of these isoforms being expressed in at least one region of the brain (Kobayashi et al., 2003, Omori and Kotera, 2007, Degerman et al., 1997, Pérez-Torres and Mengod, 2003, Bender and Beavo, 2006). PCR products were run on a 1.5% agarose gel, and product size visualised with the use of the Invitrogen 50bp DNA ladder. All primer pairs produced a product of the expected size at 35 PCR cycles (Figure 6.1).

35 cycles of PCR as detailed in 2.4.2.2 were then performed on cDNA prepared from 2 cultures of 14 d.i.v. C57BL/6 mouse primary neuronal cultures, and the products visualised on agarose gels. Clear expression of PDE3A, PDE4A, 4B and 4D, PDE7A and 7B, PDE8B and PDE10A was seen after 35 cycles of basic PCR. There was expression of PDE2A in only the first neuron preparation. There was a very weak PDE3B product observable in the second preparation. There was no product obtained using PDE4C primers, and only a very weak signal from the PDE8A primer pair (Figure 6.2). This lack of PDE4C product is consistent with reports of PDE4C in the rat brain, where it is present only in the olfactory bulb (Pérez-Torres et al., 2000). All no RT controls were negative, suggesting preparations were free from genomic contamination.

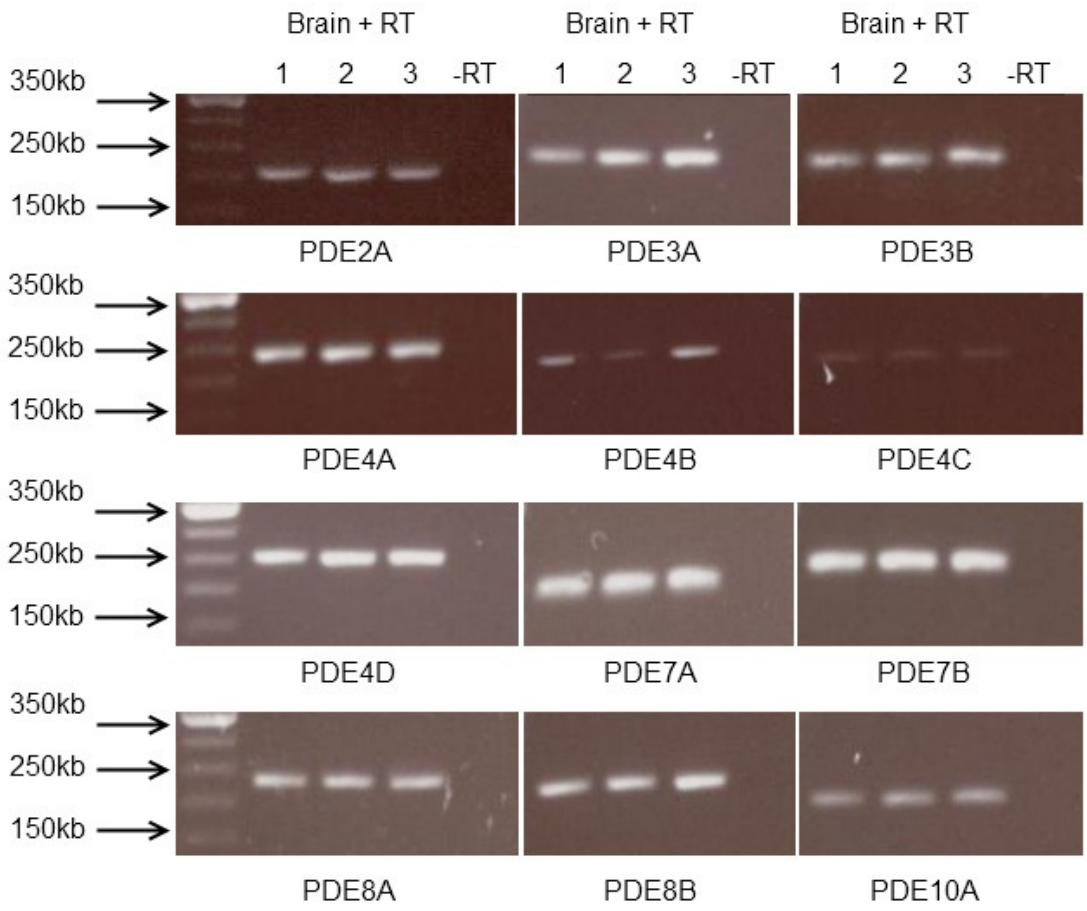


Figure 6.1: Testing of all major cAMP PDE isoform primer pairs on brain cDNA. Three cDNA preparations were produced from a single brain, roughly corresponding to anterior, mid and posterior sections (1,2 and 3 respectively). A no RT control is included as the fourth column. Expected product sizes were: PDE2A 205bp, PDE3A 230bp, PDE3b 216bp, PDE4A 240bp, PDE4B 235bp, PDE4C 232bp, PDE4D 250bp, PDE7A 200bp, PDE7B 238bp, PDE8A 229bp, PDE8B 219bp and PDE10A 200bp. At 35 cycles, all primer pairs produced a clearly visible PCR product of the correct size.

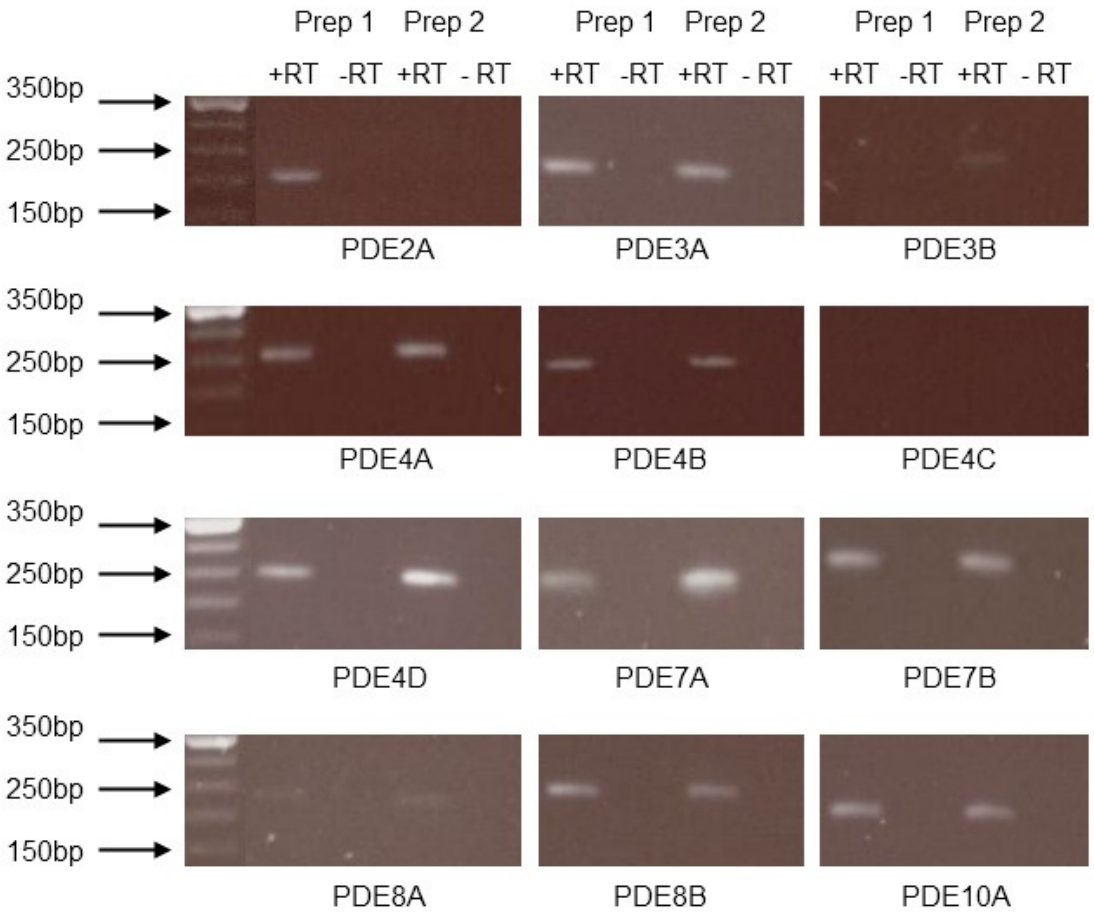


Figure 6.2: PCR products obtained from 35 cycles of basic PCR on 2 preparations of cDNA from 14 d.i.v C57BL/6 cortical neurons. A no RT control is included for each cDNA preparation.

6.2.2 Using specific inhibitors to assess which PDEs are active in primary cortical neurons

It is difficult to get an idea of the relative expression of each PDE by using basic PCR, and running the product on a gel. To try and further assess which PDEs were primarily active in these cells, specific inhibitors were applied to four sets of neuron lysates in an adapted form of the PDE assay.

The inherent difficulty in designing specific inhibitors for a set of proteins that retain rather a large amount of sequence similarity (Bender and Beavo, 2006) means that for many of the inhibitors, use at a level that inhibits the majority of the activity from their targeted PDE also has residual inhibitory effects on other PDEs. In other words, they are selective rather than specific. For example, the IC_{50} of papaverine for PDE10A is 36nM, but it is also relatively potent in inhibiting PDE2, 3 and 4, with IC_{50}s ranging from 320nM to 2.5μM (Siuciak et al., 2006). Rolipram appears to be the exception to this general principle, with an IC_{50} of less than 2μM for all PDE4 isoforms, but over 16μM for other cAMP PDEs (Chambers et al., 2006). For this reason, inhibitors were used at their IC_{50}, which should block 50% of the activity of the required PDE, without a large amount of residual activity at other PDEs. It is important to remember that IC_{50}s also vary between experiments, but are a generally useful guide. The concentrations of inhibitors used are detailed in Table 6.1.

Compound	Primary PDE	Final concentration (nM)
EHNA HCl	2	1000
Anagrelide	3	36
Rolipram	4	2000
BRL50481	7	180
Dipyridamole A	8 & 10	4500
B	10	450
Papaverine	10	36

Table 6.1 shows the concentration of PDE inhibitors used in experiment 6.2.2. All compounds are used at the IC_{50} for their primary PDE, except for dipyridamole, which inhibits both PDE8 at high doses (with an IC_{50} of 4.5μM) and is more selective for PDE10 at low doses (an IC_{50} of 450nM). In all cases

except Papaverine, IC_{50} values are obtained from the supplier, Tocris Biosciences (http://www.tocris.com/). Information on the selectivity of Papaverine, supplied by Sigma, was supplied by Pfizer (Siuciak et al., 2006, A Stillman et al., 2009).

The results obtained from this method are rather difficult to interpret. Use of each inhibitor causes a measurable decrease in total PDE activity of primary neuron lysates, suggesting that all cAMP PDEs were active (Figure 6.3). It is unclear from this experiment whether the small increase in inhibition seen at the higher concentration of dipyridamole (A) is due to inhibition of PDE8 and 10, or simply further inhibition of PDE10. The two PDE10 inhibitors, dipyramidole (at the low concentration) and papaverine inhibit PDE activity to the same extent, suggesting that the PDE10 activity of the neurons is relatively accurately estimated by this method. None of the percentage inhibition values are significantly different from those obtained from the other inhibitors (Figure 6.3). This agrees with experiments performed on unstimulated rat cortical slices in culture, where application of PDE2, PDE3 and PDE4 specific inhibitors resulted in a small but significant increase in cAMP levels of approximately the same magnitude (Suvarna and O'Donnell, 2002).

What is apparent from the raw results, and can be seen in the size of the error bars in Figure 6.3, is that the inhibition resulting from each compound can be highly variable between the four primary neuron cultures tested. For example, anagrelide, the PDE3 inhibitor inhibited 13, 22, 23, and 40% of measurable activity. EHNA HCl, the PDE2 inhibitor showed 25, 43 and 45% inhibition in three experiments, then failed to have an inhibitory effect at all in the fourth. This is consistent with PCR observations, when only one of the cDNA preparations produced a PDE2A product. The potential reasons for this variation, and the implications for the use of primary neurons in experiments such as these will be discussed at the end of this chapter. This high variability between cultures, and the selectivity, but not specificity, of the compounds used, explains why the total activity reduced by these compounds used at their IC_{50} values, is significantly

greater than 100% of total measurable activity. Although this makes comparisons between the activity of different isoforms difficult to make, it does show clear measurable activity from all cAMP PDE isoforms except PDE8, which this experiment was unable to resolve. It may have been advisable to use an IBMX control to eliminate all PDE activity in this experiment.

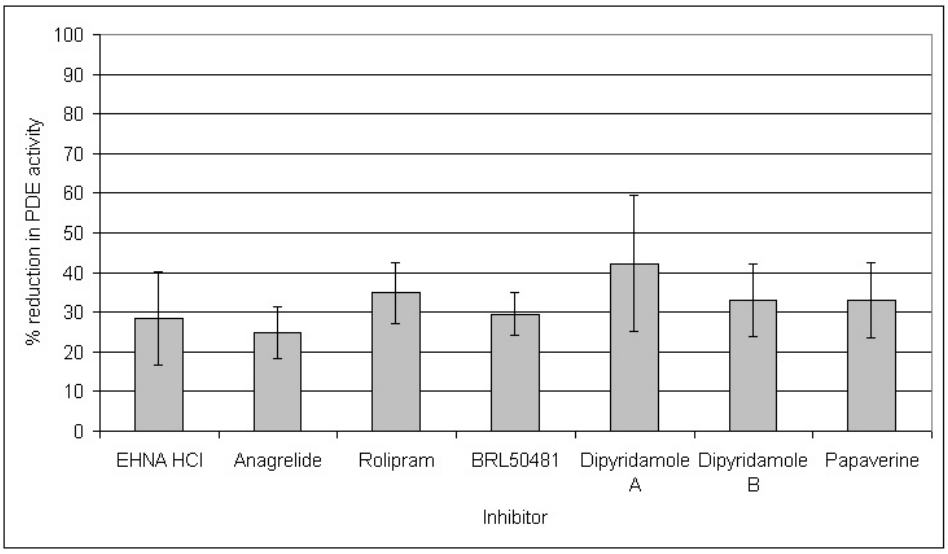


Figure 6.3: A graph detailing the percentage inhibition of PDE activity resulting from the use of a series of selective inhibitors on primary cultured neuron lysate. None of the differences in activity seen are significant after 4 experiments. There was a large amount of variability observed between neuron cultures, as can be seen in the large error bars.

6.2.3 Endogenous PDE4B and PDE4D colocalise with DRD1 and NR1 in dendritic spines of primary cortical neurons

As all neuronal cultures tested showed significant inhibition of PDE activity in response to treatment with rolipram, immunocytochemistry was performed to examine the localisation of PDE4B and PDE4D, to discover if they are located close to the receptors of interest: dopamine type I and NMDA. Primary antibodies were pan PDE4B and pan PDE4D raised in sheep, a kind gift from Miles Houslay, University of Glasgow, and two

commercial antibodies: D6692 (Dopamine Type I receptor, Sigma) and MAB363 (NR1 subunit of NMDA receptor, Chemicon).

Colocalisation of both PDE4B and PDE4D was observed with DRD1 and NR1. While there is some colocalisation in the cell body, most of the signal is seen along the lengths of the dendrites, where dendritic spines are beginning to develop (Figures 6.4-6.7). DISC1 has previously been observed to colocalise with PSD95 in dendritic spines of mature (21 D.I.V.) primary hippocampal neurons (Bradshaw et al., 2008). Dendritic spines are sections of membrane which protrude from dendrites to receive an input, typically from a single excitatory synapse. They are very diverse structures, containing a large and dynamic collection of proteins, and form the major excitatory surface of neurons (90% of excitatory synapses terminate on them). Dysfunction has been implicated in a wide array of psychiatric and neurological disease (see Nimchinsky et al., 2002 for an in depth review). The presence of PDE4B and PDE4D in the same dendritic spines as these two excitatory receptors suggests that phosphodiesterase activity changes may play a key role in downstream signalling from these receptors. This is to be expected, as temporal and spatial control of cAMP fluxes is key for coding downstream signalling (Smith and Scott, 2006). By proxy, DISC1, due to its presence in spines and the changes it can cause in PDE4 activity in SHSY5Y cells, may also be an important mediator of downstream signalling responses.

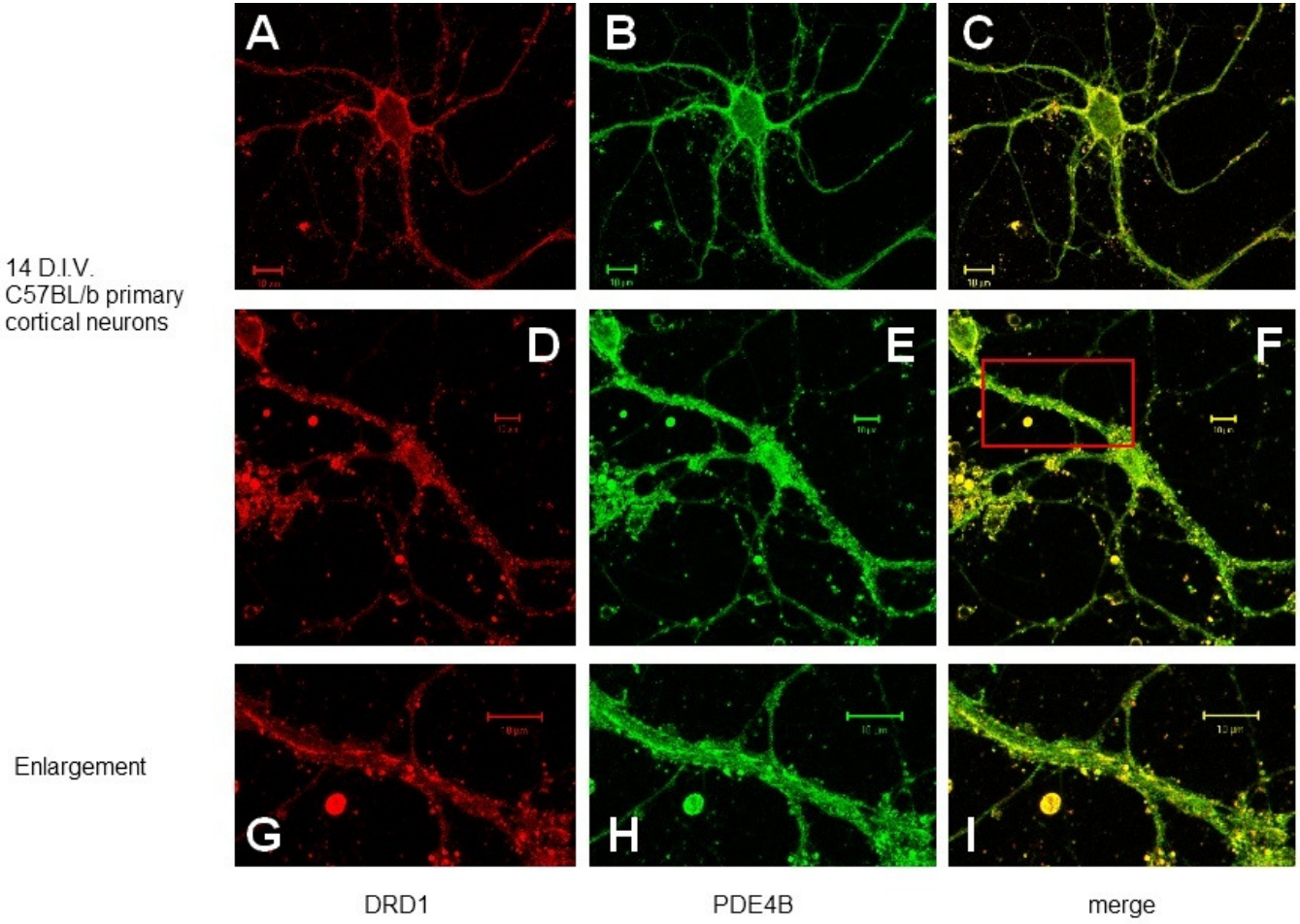


Figure 6.4 A-F shows colocalisation of DRD1 and PDE4B in 14 D.I.V. primary cortical neurons. G-I shows an enlargement of the highlighted area of F, which depicts a high level of colocalisation in developing dendritic spines. In all figures 6.4 to 6.7 scale bars show 10µm.

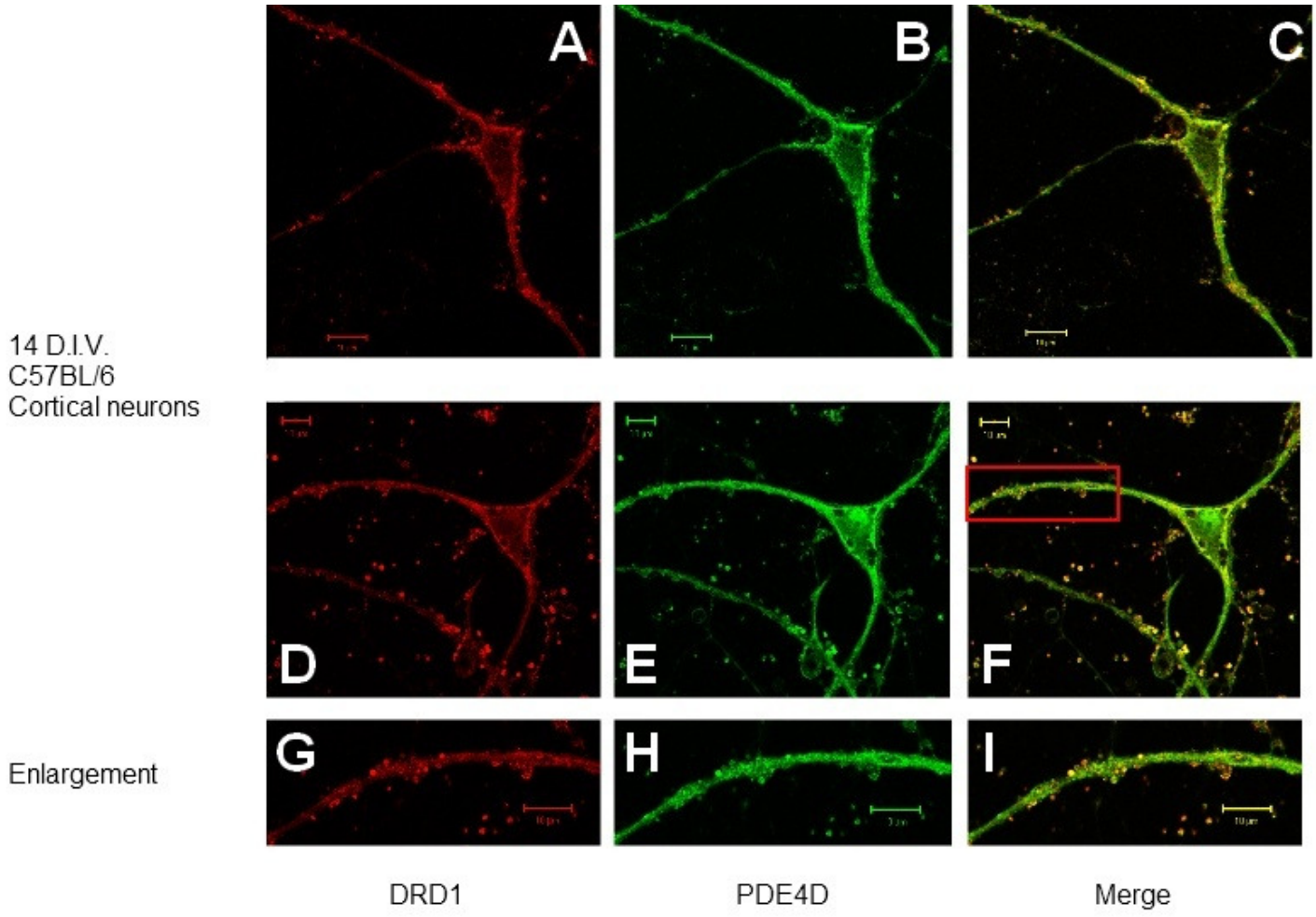


Figure 6.5 A-F shows colocalisation of DRD1 and PDE4D in 14 D.I.V. primary cortical neurons. G-I shows an enlargement of the highlighted area of F, which depicts a high level of colocalisation in developing dendritic spines.

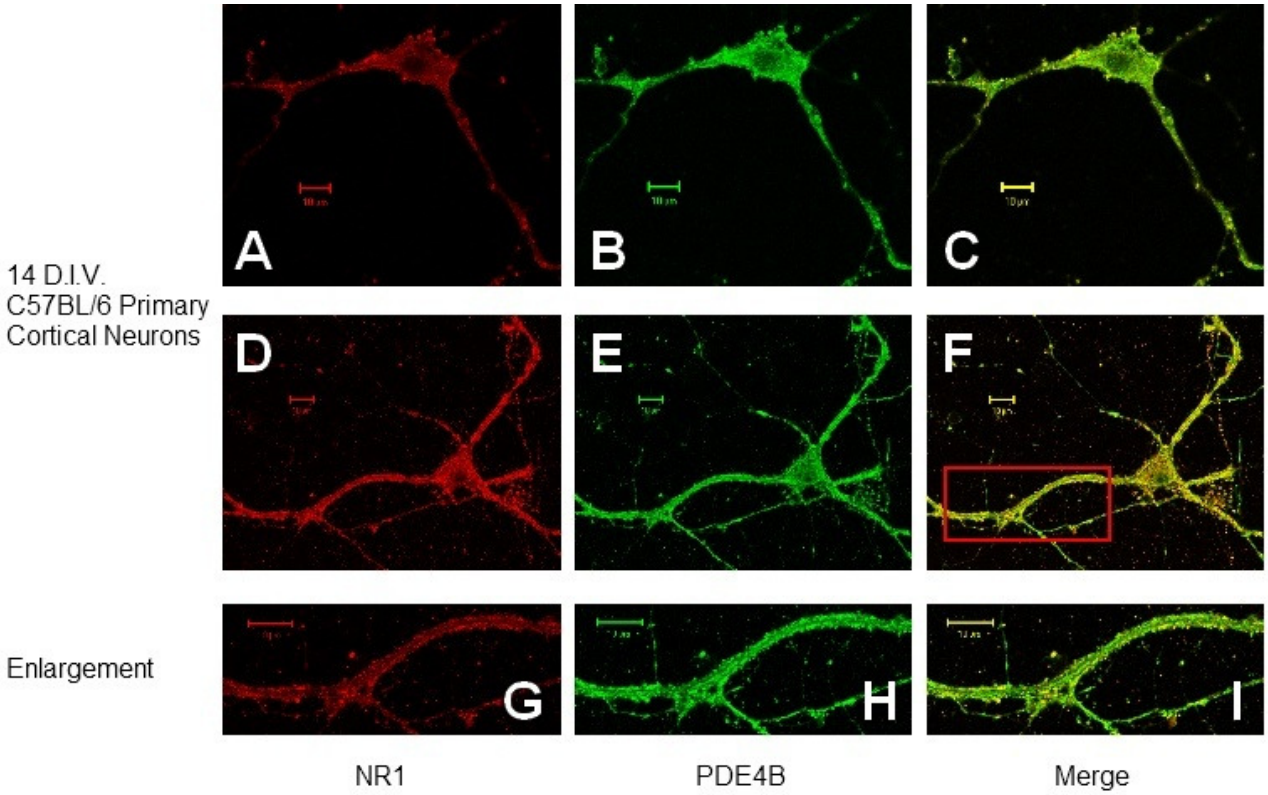


Figure 6.6 A-F shows colocalisation of NR1 and PDE4B in 14 D.I.V. primary cortical neurons. G-I shows an enlargement of the highlighted area of F, which depicts a high level of colocalisation along the exterior surface of the dendrites, where spines will develop.

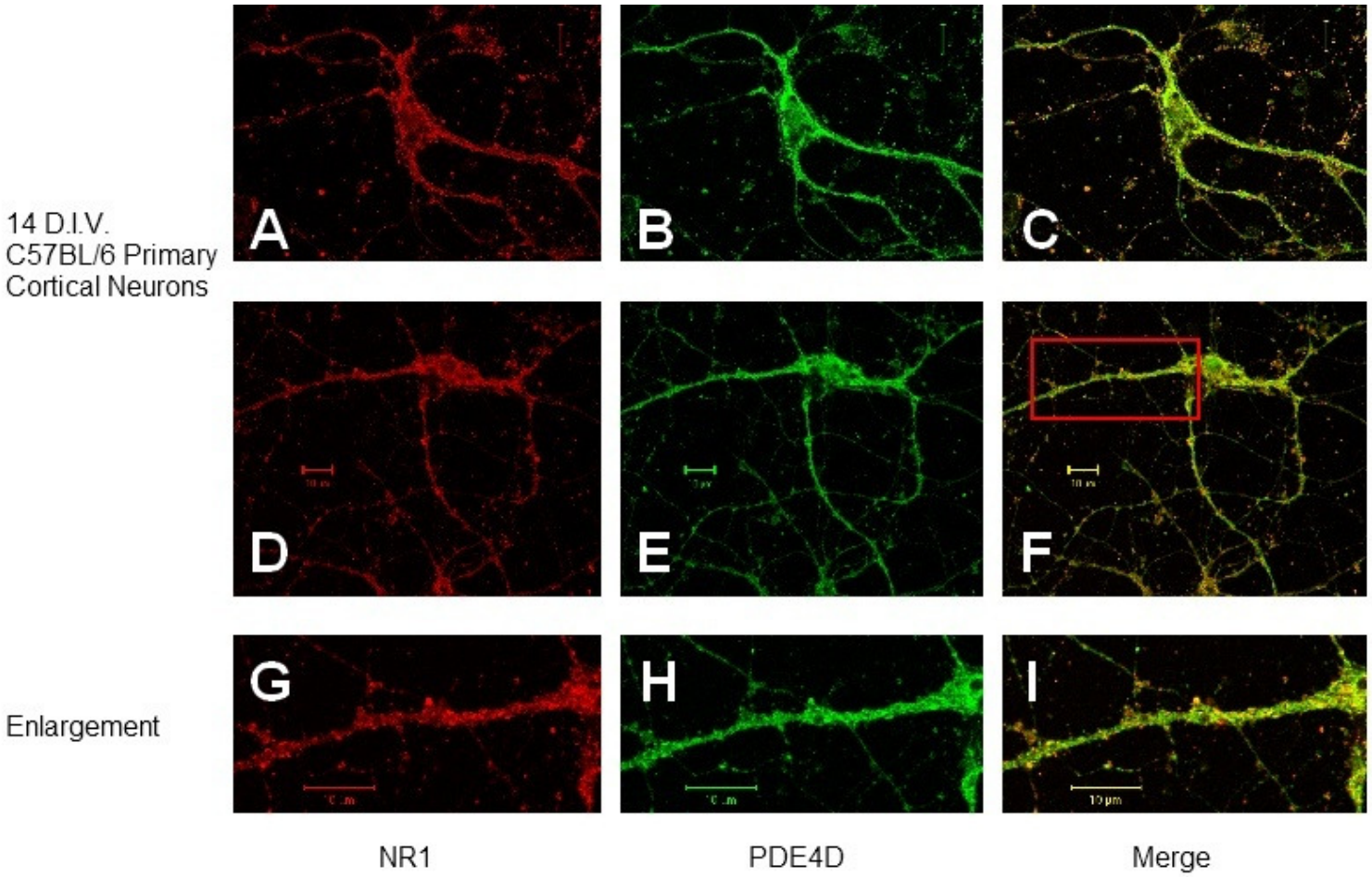


Figure 6.7 A-F shows colocalisation of NR1 and PDE4D in 14 D.I.V. primary cortical neurons. G-I shows an enlargement of the highlighted area of F, which depicts a high level of colocalisation in developing dendritic spines.

6.2.4 Treatment of 14 D.I.V. primary cultured CD1 neurons with NMDA causes an increase in PDE4 activity.

The inhibition of a significant proportion of total cortical neuron PDE activity by rolipram, and the finding that PDE4B and D colocalise with DRD1 and NR1 in dendritic spines, suggests that changes in PDE4 activity may be acting downstream of receptor agonism to mediate downstream cellular responses. In rat cerebral cortical cultures, treatment with IBMX, a non specific PDE inhibitor, and rolipram, led to 3-7.5 fold increase in cAMP in response to NMDA treatment (Suvarna and O'Donnell, 2002). This is indirect evidence that PDE4 is the primary PDE active in breaking down cAMP generated by NMDA agonism. To investigate this possibility, primary cortical neurons were grown in culture for 14 days, then supplement deprived for 4 hours. An initial concentration curve was performed, and 15μg of protein chosen for assays of neuronal lysate (Figure 6.8A). A combination of 10μM NMDA and 100μM glutamate, known to elicit measurable calcium transients within 7 minutes of beginning treatment in ND7 cells (Sanchez-Martin et al., 2006), was applied to neuronal cultures for 2 or 5 minutes as an initial trial. In hippocampal slice cultures as previously described, an NMDA mediated increase in cAMP concentration was apparent at 1 and 5 minutes of treatment, but had returned to baseline by 20 minutes (Chetkovich and Sweatt, 1993). Cultures were then lysed and PDE assayed as previously described. To control for the potential that disturbance of the media or changes in temperature may alter PDE activity in these highly sensitive cells, I used two vehicle treated timepoint controls as a comparison, not a zero timepoint.

Treatment of primary cortical neurons with a combination of NMDA and glutamate induced a significant increase in PDE4 activity at both 2 and 5 minutes of treatment (Figure 6.8B). This was despite PDE4 being in the minority of measurable PDE4 activity in these CD1 primary neurons, ranging from undetectable to 44% of measurable activity in vehicle treated neurons (a mean of 18% +/-4), and there being a wide variety of measurements obtained. There were no significant changes observed in non-PDE4 activity (Figure 6.8C)

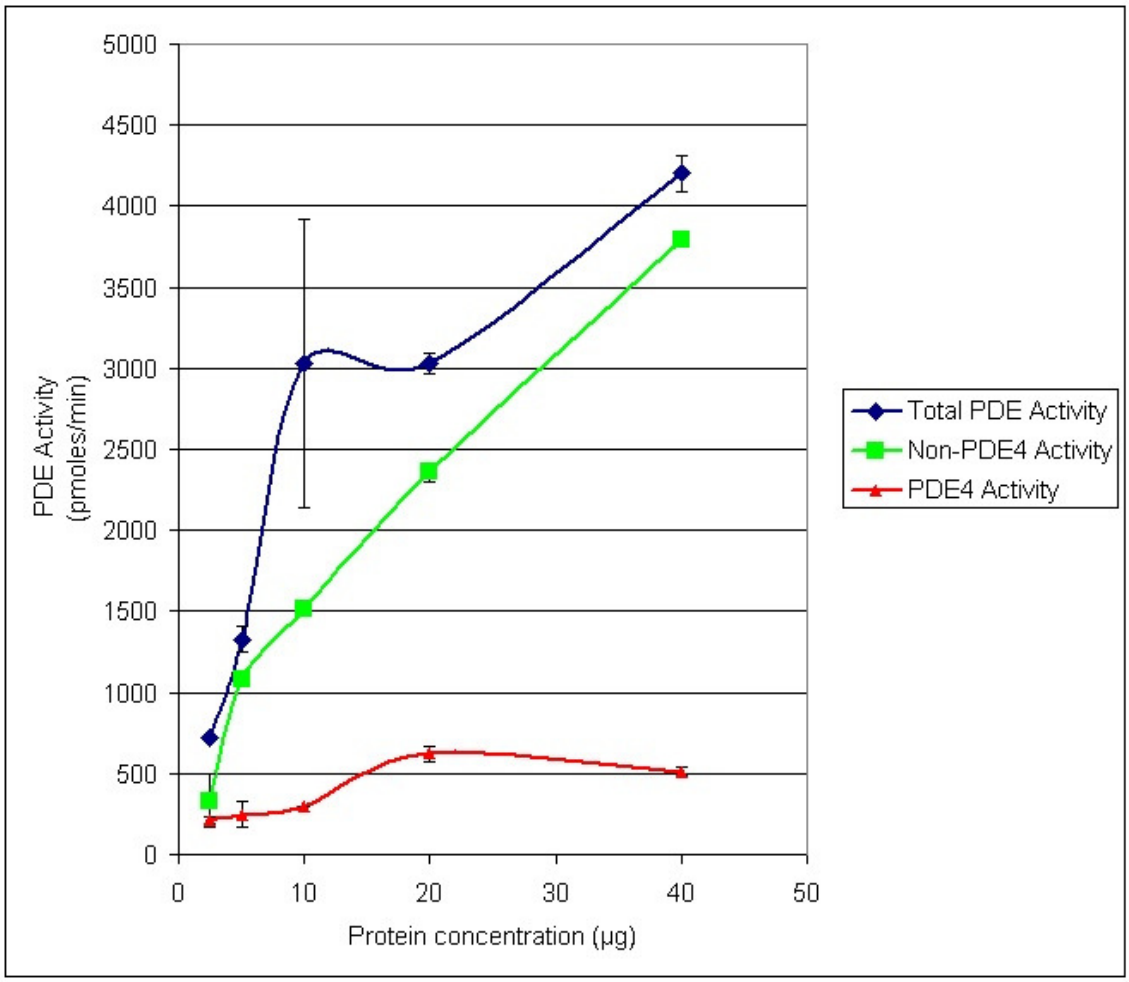


Figure 6.8A shows a concentration curve determining PDE activity in lysate from primary cortical neurons. The PDE4 component forms a smaller proportion of the measureable activity in these cells. A protein concentration of 15µg was chosen for the following assays.

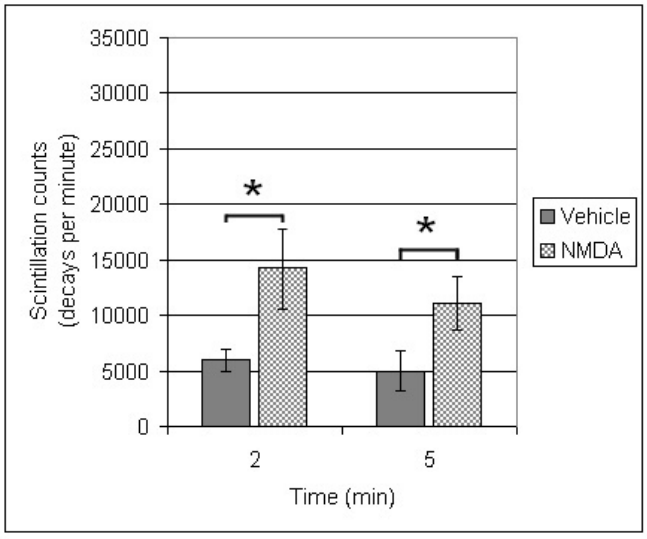


Figure 6.8B: Graph to show the significant increase in PDE4 activity on treatment of primary cortical neurons with a combination of NMDA and Glutamate. In both parts of the figure, values are shown as absolute, measured in scintillation counts, as opposed to relative levels as in 2 cases PDE4 activity was undetectable in vehicle treated cells. * n=11, p=0.04. The mean PDE4 activity in the vehicle treated 2 minute control is 29.9pmoles/mg/min.

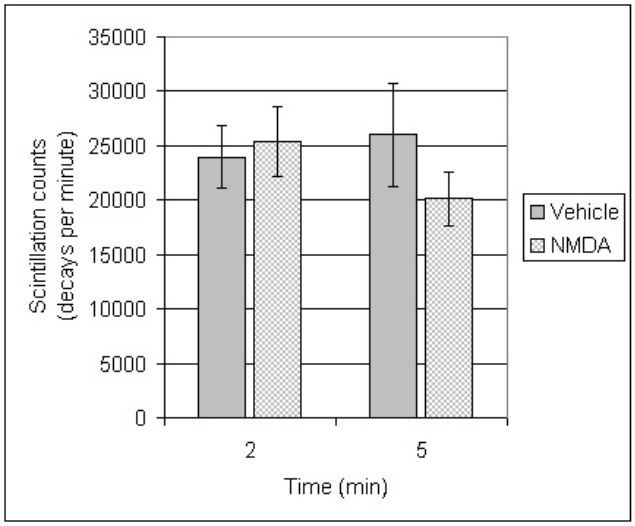


Figure 6.8C: Graph to show there is no change in non-PDE4 activity on treatment of primary cortical neurons with a combination of NMDA and Glutamate. The mean non-PDE4 activity in the 2 minute vehicle treated control is 119.4 pmoles/mg/min.

6.2.5 Treatment of 14 D.I.V. primary cultured CD1 neurons with a DRD1 selective agonist results in an increasing trend in PDE4 activity.

As previously, CD1 primary neurons were grown for 14 days in vitro, before being supplement starved for 4 hours. Neurons were exposed to a DRD1 agonist for 2 or 5 minutes. The DRD1 agonist in question was A68930, which is highly selective for type 1 dopamine receptors at nanomolar concentrations (DeNinno et al., 1991). A final concentration of 1nM was employed in this experiment. Unfortunately, due to limitations in the number of mice available, only 6 cultures were available for treating and PDE assaying. After six experiments, there was a trend which was approaching significance ($p=0.08$, $p=0.16$ for 2 and 5 minutes respectively) towards an increase in PDE4 activity at both timepoints (Figure 6.9A). At both timepoints, 4 out of 6 experiments showed an increase in PDE4 activity, 1 showed no change, and 1 a decrease. Once more, there was a large amount of variation in the magnitude of the response to DRD1 agonism, ranging from a decrease of 5% to an increase of 30% at 2 minutes (8% average increase +/-5), and a decrease of 8% to an increase of 56% at 5 minutes (13% average increase +/-10). Stimulation of PDE4 via DRD1 agonism can therefore not be ruled out, but certainly requires further investigation before confirmation. The magnitude of the response to DRD1 agonism, which is coupled to and activates adenylyl cyclase, is significantly lower than that observed by direct activation of adenylyl cyclase by forskolin in SHSY5Y cells.

Similarly, there is a trend towards a decrease in the non-PDE4 component of the response to A68930 (Figure 6.9B), but again this is non significant ($p=0.17$, $p=0.1$ for 2 and 5 minutes respectively) despite this being the largest component of measurable activity. It is possible that differential effects of stimulation and inhibition are occurring on the different PDEs that make up this component of the response, which may cloud changes in particular isoforms.

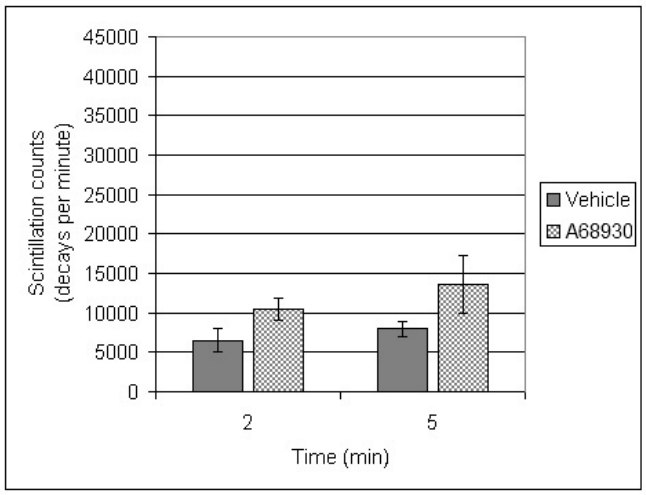


Figure 6.9A: Graph to show the trend towards an increase in PDE4 activity on treatment of primary cortical neurons with A68930, a DRD1 selective agonist. As previously, values are shown as absolute, measured in scintillation counts. Mean PDE4 activity in the 2 minute vehicle treated control is 41.6pmoles/mg/min.

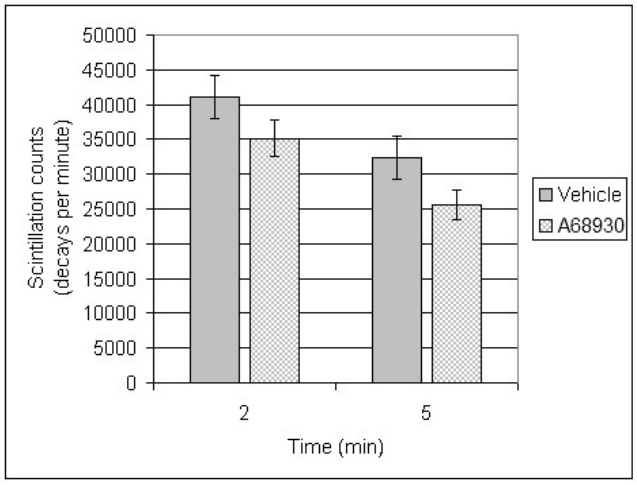


Figure 6.9B: Graph to show the trend towards a decrease in non-PDE4 activity on treatment of primary cortical neurons with A68930, a DRD1 selective agonist. Mean non-PDE4 activity in the vehicle treated 2 minute control is 191.0pmoles/mg/min. Due to the high counts in this assay, readings may occur in the non-linear range.

6.2.6 Inhibition of GSK3β in primary cortical neurons has no effect on PDE4 activity

As previously, primary cortical neurons were cultured for 14 days in vitro then supplement deprived for 4 hours. Three cultures were treated for 30 minutes with vehicle, 10mM LiCl, 3μM of the GSK3β specific inhibitor SB21673, or a combination of the two compounds, then PDE activity assayed as previously. Unlike observations made in SHSY5Y cells, there was no measurable effect of GSK3β inhibition on PDE4 specific activity (Figure 6.10A). Once more, PDE4 specific activity was a minor component (18% +/-3) of the total activity measured. It may simply be that the PDE assay is not sensitive enough to detect subtle changes to such a low basal rate of activity. Because neurons are highly differentiated cells, there is also the potential that GSK3β inhibition is altering PDE4 activity, but only at specific locations within the cell. This technique is likely not sensitive enough to detect such small scale, but potentially important changes.

There was however, a trend towards inhibition of the non-PDE4 component of the measurable activity, which was approaching significance after 3 experiments (Figure 6.10B). In chapter 5 I alluded to the potential for GSK3β inhibition having an effect on one, or more of the other PDEs. It would seem that this experiment also highlights this possibility. The observed decrease was more variable than that observed with PDE4 in SHSY5Y cells (ranging from a 4-43% decrease in activity), but occurred consistently throughout all three experiments. The proportion of activity inhibited by the range of selective PDE inhibitors in experiment 6.6.2 also varied widely between neuron cultures. It may be therefore, that this experiment is not detecting an inconsistent change in PDE activity in response to GSK3β inhibition, but varying expression of the PDE that is being inhibited. Without knowing more about the mechanism of action of GSK3β on PDE4 – whether the effect is direct or via mediating proteins, it is difficult to speculate on which of the other PDEs may also be affected.

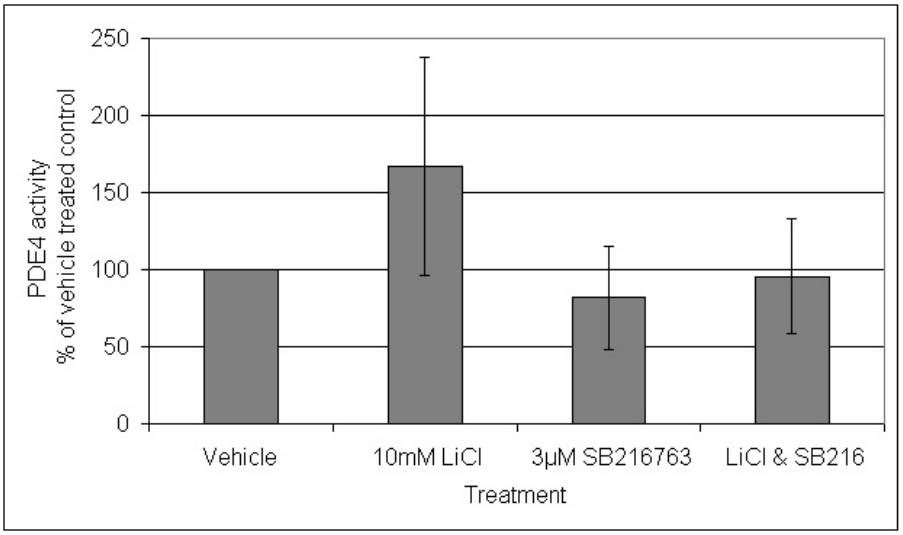


Figure 6.10A: Treatment of primary cortical neurons with 10mM LiCl and 3μM SB216763 has no significant effect on PDE4 activity. Mean PDE4 activity in the vehicle treated control is 23.5pmoles/mg/min.

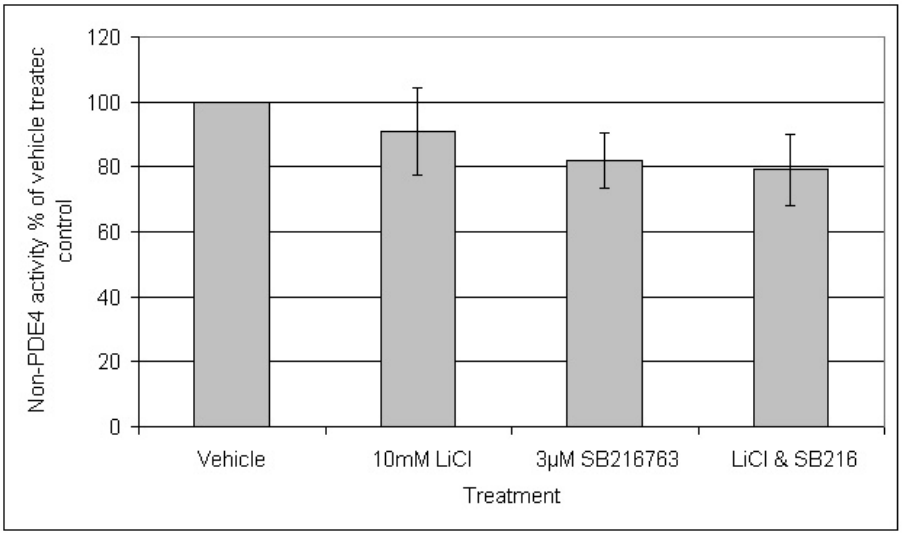


Figure 6.10B: Treatment of primary cortical neurons with 10mM LiCl and 3μM SB216763 causes a decrease in non-PDE4 activity which trends towards significance after 3 experiments. Mean non-PDE4 activity in the vehicle treated control is 111.8pmoles/mg/min.

6.2.7 Standard PDE assays detect no difference in PDE4 activity in DISC1 missense mutant mice

In his 2007 paper, Clapcote describes two missense DISC1 mutant mice, one with a "depressive like" phenotype (Q31L) and one with a "schizophrenic" like phenotype (L100P). These are reviewed in greater detail in the introduction. When mouse DISC1 bearing one or other of these mutations is coexpressed with PDE4B1 in HEK293 cells, binding to PDE4B is reduced. Immunoprecipitated PDE4B from Q31L mouse brain lysate, is 50% less active than that immunoprecipitated from wild type mouse brain lysate, despite there being no changes in PDE4B protein expression. I therefore wanted to test whether my experimental system could detect this change in PDE4B activity in total lysate from primary cortical neurons, as this would allow the potential for detecting changes in response to compounds on a background of mutant DISC1.

To prepare mice for this experiment, homozygous crosses were performed with genotyped wild type and mutation carrying C57BL/6 mice, to avoid the need for genotyping pups. Matings were timed and primary neurons prepared as detailed in 2.2.2. Neurons were cultured in vitro for 14 days, supplement deprived for 4 hours to mimic drug treatment experiments, then lysates prepared. Concentration curves using 5, 10, 15 and 20μg protein per assay tube were produced, assaying matched sets of lysates, ie. one Q31L mutant with one WT from the Q31L breeding colony. It was not possible to use wild type litter mates, as the genotype of the pups had to be known before neuron preparation, but mice were as closely matched as possible.

There were no observable differences in PDE4 activity in either the Q31L or the L100P experiment (Figure 6.11 and 6.12). PDE4 activity did not produce a clean concentration curve, as it formed such a small proportion of measurable activity that there was a large amount of error inherent in the assay. Non-PDE4 activity was also very closely matched in L100P mice. In Q31L mice, there was a trend towards increased activity in the non-PDE4 component. After three experiments, this trend was approaching significance. It is possible that although I could not measure a difference in PDE4 activity in these mice,

this increase in non-PDE4 activity could be a compensatory mechanism for decreased PDE4B activity, as was seen in brain lysates. There was no evidence of the specific 50% decrease in PDE4B activity in Q31L mice reported by Clapcote *et al,* 2007, when PDE4B was immunoprecipitated and activity assayed. It is possible that this only occurs in the mice as they are living behaving entities, responding to external stimuli. Changes in PDE4B activity may only be revealed in response to stimulation of neurons in response to this activity, which is not occurring in these in vitro cultures. It is also possible that activity of other PDE4s, PDE4D for example, may compensate for a decrease in PDE4B activity. This experiment measuring total PDE4 activity, and not PDE4B specific activity from immunoprecipitated protein, would fail to detect changes if such compensation had occurred. Due to the small absolute values obtained for PDE4 activity in this assay, small decreases in activity will be difficult to detect. However, this system is able to detect activation of PDE4 in response to NMDA or DRD1 stimulation, as this process increases the proportion of measurable activity.

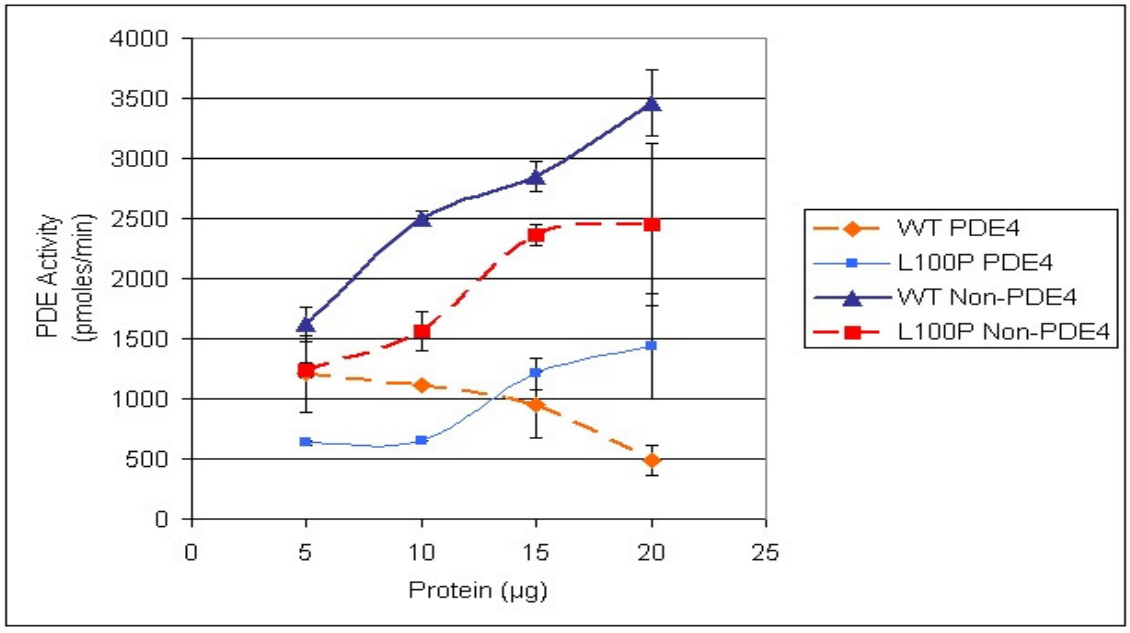


Figure 6.11: Concentration curves showing PDE4 and non-PDE4 activity in L100P background and mutant mice.

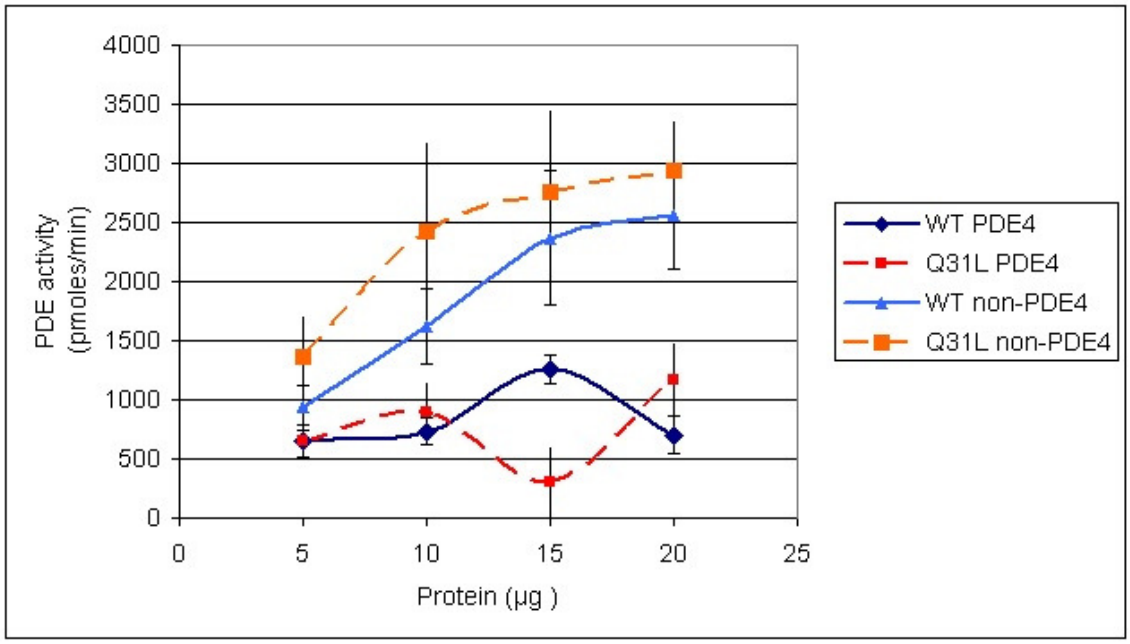


Figure 6.12: Concentration curves showing PDE4 and non-PDE4 activity in Q31L background and mutant mice. There is a trend towards an increase in non-PDE4 activity, but this is non-significant.

6.3 Discussion

Earlier in this thesis I defined the use of SHSY5Y cells as a model for assessing PDE4 activity in a cultured cell line derived from a neuronal environment. SHSY5Y cells were ideal for such a purpose, as their basal level of PDE activity is high, and almost entirely PDE4 specific, maximising the potential for detecting changes to activity resulting from chemical or genetic manipulations. The aim of the experiments in this chapter was to further define the PDE profile of primary cultured neurons, to ascertain whether they could be used in similar experiments.

A striking point to be taken from these experiments is the much wider variation inherent in using neurons when compared to a cell line. There was a large amount of variation between experiments and different neuron preparations in drug treatment experiments, and in the PCR experiment, PDE2A and PDE3B were only present in one of the two cDNA preparations tested. In the drug treatment experiments, although NMDA agonism

caused an increase in PDE4 activity in all but one of the cultures tested, the magnitude of the response varied greatly, requiring a large number of experiments to be undertaken before reaching significance. Of course, this variation is not surprising, given the multiple levels before and during neuron preparation where variation can be introduced. I have tried to summarise these sources of variation, and how they may be minimised in table 6.2. As one can see, the majority of these causes of variation can be completely negated by using a clonal cell line such as SHSY5Y to give more consistent results.

While attempts were made to minimise the effect of these sources of variation as much as possible, as detailed in the third column of Table 6.2, it was impossible to eliminate all sources using the current experiment design. It was also difficult to predict from the literature what the timescale of a downstream response to receptor agonism would be. Increasing the throughput of the assay to perform more time points or, developing a real time method of visualisation in single cells would be a superior approach. Increasing the throughput of these experiments would be relatively difficult, as one always wishes to minimise the number of animals used, and major new equipment would have had to be bought to increase the throughput of the PDE assay (which has been successfully performed in a 96 well format).

While the low throughput PDE assay is able to consistently detect an increase in PDE4 activity in neurons in response to an activating treatment, it may not be sufficiently sensitive to detect a decrease in activity if the basal activity of a specific PDE forms a small proportion of measurable activity. For example, it was unable to detect a change in PDE4 activity in response to lithium treatment. Finally, this assay, and the production of a whole cell lysate only allows for detection of a rather large scale event across the cell. It is possible that while PDE4s are being activated in close proximity to the receptor, they are being turned off elsewhere. Thus, while I am convinced that the

Stage of preparation process	Means of introducing variation	Means of minimising variation
Mouse breeding	There is some natural genetic variation between animals.	Inbreeding of mouse lines. High number of biological replicates
Use of primary culture	The brain is a hugely heterogenous tissue with many cell types.	Use B27 supplement, Glutamax and Neurobasal medium to predispose towards neuron formation
	There are a large number of different types of neuron in any primary culture	Not possible in this experiment
	Neurons are highly differentiated cells with many functional compartments	Not possible in this experiment
Dissection	It is difficult to consistently dissect exactly the same brain areas	Practised dissection with sharp forceps. The same person performs all dissections.
Culture	Some preparations grow better than others, resulting in differences in neuron density	Not possible in this experiment
	Differences in neuron density can lead to changes in the amount of connectivity between cells, and change expression profiles	Count cells before seeding them. Practised dissection to reduce preparation time.
	Stress to cells during culture and drug treatment	Use a well maintained clean incubator. Employ a standard protocol for drug treatment.
PDE Assay	Absolute values vary significantly between experiments	Use proportional results where possible, carefully control the temperature throughout the experiment.

Table 6.2: A summary of the different levels of an experiment involving primary neurons, where variation may arise from during this process, and how it can be minimised in my experimental design.

significant activity increases I have measured in response to NMDA are real, and that the non-significant trend in response to the DRD1 agonist A68930 are similar, this experimental approach was suboptimal, but all that I was personally able to undertake in the time available. For this reason, if others were to pursue this avenue of research, I would recommend a different approach, particularly one with greater spatial resolution, such as the one detailed below.

Unsuccessful attempts were made to assay neurons using the AlphaScreen to increase throughput, but the cAMP signal obtained was highly inconsistent from well to well. It is likely that plating neurons in the 384 well plates was not conducive to even growth, but as they were opaque white it was not possible to investigate this. To avoid transfection of post mitotic primary neurons in culture, a generally quite inefficient and damaging process, the most elegant way to create a cAMP or PDE biosensing population of primary neurons is to create transgenic mice from which neurons can be prepared and analysed. Calebiro *et al* (2009) used this approach, creating transgenic mice constitutively expressing an Epac based FRET cAMP sensor (YFP/CFP). These mice appeared to be physiologically normal throughout development, despite expression of the sensor in most tissues. Primary neurons were isolated, and stimulated with isoproterenol (a beta adrenergic receptor agonist), which induced a short lived decrease in the YFP/CFP ratio. This response was more robust when rolipram was added to cultures, demonstrating that PDE4 was responsible for inactivating the cAMP flux resulting from isoproteronol treatment (Calebiro et al., 2009). The beauty of a system such as this is that is provides spatial and temporal resolution of cAMP fluxes in single cells, without the need for transfection of neuron cultures. It also enables the researcher to choose which cells to report from, negating some of the problems of mixed cell types in primary cultures. The ratiometric read out of fluorescence allows for quantification of the cAMP flux, meaning that there is the potential for miniaturisation of such a system to a well plate format.

The biochemical PDE activity assay has painstakingly provided evidence for a role for PDE4B and 4D acting downstream of DRD1 and NR1 signalling. To add to this evidence, colocalisation of endogenous PDE4B and 4D with DRD1 and NR1 was clearly visualised along dendrites in structures resembling developing dendritic spines. These structures form the major excitatory structures of neurons, and abnormalities in their survival and morphology have been implicated in schizophrenia, normal ageing and mental retardation (reviewed in Nimchinsky et al., 2002). Post mortem studies are difficult however, as dendritic spines are highly plastic structures, capable of changing morphology in a space of minutes (Nimchinsky et al., 2002). One DISC1 truncation mouse model has a decreased number of dendritic spines on granule cells in the dentate gyrus (Kvajo et al., 2008), while an alternative DISC1 knock down model shows accelerated formation of dendritic spines (Duan et al., 2007). Thus, a role for the PDE4s and DISC1 in modulating cAMP fluxes in dendrites, and the downstream response to them is clearly possible.

This functional linking of PDE4, a risk factor for schizophrenia, with two of the major pharmacological pathways implicated in the pathogenesis of the disease is tantalising. The evidence from chapter 5 that the major susceptibility gene DISC1 may act to modulate PDE4 fluxes downstream of increased adenylyl cyclase activity adds interest to this link. Furthermore, unpublished evidence from the Millar lab suggests an interaction between DISC1, the Yotiao fragment of AKAP9, and the NR1 subunit of the NMDA receptor (Mackie *et al*, unpublished). The theory of glutamatergic hypofunction in schizophrenia stems from two types of evidence. Firstly, short term administration of the non competitive NMDA receptor antagonists PCP and ketamine to healthy humans results in a psychomimetic state that models aspects of the positive, negative and cognitive symptoms of schizophrenia (Javitt and Zukin, 1991, Lahti et al., 1995, Krystal et al., 1994). Hospital departments report that it is almost impossible to distinguish a schizophrenic patient from a PCP drug user (Javitt and Zukin, 1991). In schizophrenic patients, even those who are stable, PCP administration results in psychotic episodes that recapitulate the features of earlier non-drug induced psychosis. These symptoms can

persist for weeks after drug administration (Javitt and Zukin, 1991). The effects on the cognitive component of schizophrenia are less clear. Deficits have been reported in the Wisconsin Card Sorting Test (Krystal et al., 1994) and verbal encoding paradigms, while spatial working memory appears to remain intact (Rowland et al., 2005).

Secondly, agonists that bind the glycine modulatory site of the NMDA receptor to potentiate activity, such as glycine, D-serine and D-cycloserine, improve positive and negative symptoms when used as adjuncts to conventional antipsychotic medication (Tsai et al., 1998, Goff et al., 1999, Heresco-Levy et al., 1999). More recently, a number of schizophrenia susceptibility genes have been highlighted that function in glutamate related pathways. These include DAAO, which metabolises D-serine, G72, which activates DAAO, NRG1 which regulates expression of NMDA receptors through ErbB4 receptors, and RGS4 which acts as a negative regulator of GPCR activity, including metabotropic glutamate receptors (Harrison and Owen, 2003). Glutamatergic neurons form the connections between the frontal cortex, limbic system, basal ganglia and thalamus, all brain regions which have been linked to schizophrenia (Moghaddam, 2003), and have been strongly implicated in axon guidance, synaptic pruning and LTP (Meador-Woodruff and Healy, 2000). Early trials of highly selective mGluR2/3 receptor agonists showed inhibition of PCP induced hyperlocomotion in mice, and have shown promising improvements in both the negative and positive symptoms of schizophrenia patients (Patil et al., 2007).

Despite this, evidence is weak for NMDA receptor expression changes in schizophrenic patients (Clinton and Meador-Woodruff, 2004), although decreased activating phosphorylation at Ser897 of NR1 has been found in hippocampi from schizophrenic patients (Emamian et al., 2004b). One of the main criticisms of this theory is that glutamatergic signalling is so widespread throughout the brain that larger scale disorders than schizophrenia would result from disruptions to the system (Moghaddam, 2003). If PDE4 and DISC1 are involved in the fine tuning of cAMP fluxes downstream of NMDA receptors, it may be that they only associate with a subset of receptors in specific regions

of the brain. Thus if PDE4 or DISC1 are functioning sub optimally, due to presence of a risk SNP for example, this offers an explanation as to why symptoms may be more localised and mild than would be expected for a large scale glutamatergic disruption.

I have also shown a trend towards PDE4 activation on agonism of DRD1 receptors. These receptors form the majority of dopaminergic receptors in the PFC, and are located on dendritic spines, particularly on distal dendrites (Weinberger and Laruelle, 2001). The hyperactive dopaminergic theory of schizophrenia was originally hypothesised after two observations, the first from studies on the targets of first generation antipsychotic drugs, and the second that dopamine enhancing drugs can generate psychosis (Abi-Dargham, 2005). First generation antipsychotics were found to inhibit release of dopamine from rat striatal slices, and the dose required for 50% inhibition correlated with clinical doses used in patients (Seeman and Lee, 1975). This effect was attributed to blocking of what was then termed the "neuroleptic" receptor with an IC_{50} related to neuroleptic potency (Seeman et al., 1976). This receptor was later defined as the type II dopamine receptor (DRD2), and until the trials with the mGluR2/3 agonist (Patil et al., 2007), all licensed anti-psychotic drugs possessed activity at this receptor (Abi-Dargham, 2005).

The second strand of the evidence comes from observations that dopamine releasing drugs such as amphetamine and L-DOPA can lead to psychotic episodes in healthy individuals (for a detailed review see Abi-Dargham, 2005). To summarise the contents of this review, repeated exposure to amphetamine can lead to paranoid psychosis, hallucinations and thought disorder. Schizophrenic patients are also more sensitive to induction of psychosis by low concentrations of amphetamine than healthy individuals. The major criticism of this early theory however, was that while it successfully explained the positive symptoms of schizophrenia, it failed to explain the negative symptoms. It was already known that dopaminergic tone in the PFC exerts an inhibitory effect on dopaminergic transmission in the striatum (Abi-Dargham, 2005, Weinberger and Laruelle, 2001). Thus a dual theory was proposed of hypoactive dopaminergic tone

in the PFC, which leads to dysregulation and hyperactivity of striatal dopamine (Abi-Dargham, 2005).

Since this dual theory was proposed, imaging studies have strengthened the hypothesis, and further evidence has integrated this theory with the glutamatergic theory. In their 2001 meta-analysis, Weinberger and Laruelle revealed a small but significant increase of DRD2 receptor expression, and an increase in DOPA decarboxylase expression in the striatum of schizophrenic patients. Schizophrenic patients also showed more variation in expression of those components then healthy controls. They also saw a significant increase in dopamine release in the striatum, as inferred by studies of amphetamine induced radiotracer binding to DRD2. Dopamine depletion studies revealed a higher DRD2 occupancy in schizophrenic patients, which was predictive of a good response to 6 weeks of antipsychotic treatment (Weinberger and Laruelle, 2001). Since this analysis, increased availability of DRD1 has been demonstrated in the dorso-lateral PFC (DLPFC) in schizophrenic patients, which is unrelated to antipsychotic status and associated with poor performance in the N-back working test of working memory and executive function (Abi-Dargham et al., 2002).

This functional link between the PFC and striatum allows for integration of both the dopaminergic and glutamatergic theories of schizophrenia. The PFC influences striatal dopamine through an activating glutamatergic input, and an inhibitory GABAergic input (Weinberger and Laruelle, 2001, Mignon, 1996). There is currently widespread controversy over whether the principle deficit in schizophrenia is glutamatergic, dopaminergic, or indeed GABAergic. The complexities of these arguments fall outside the scope of this thesis (Seeman, 2009, Kim et al., 2009a, Gaspar PA et al., 2009)but it is clear that dysregulation of both these components leads to symptoms that recapitulate a number of the symptoms of schizophrenia, and that small disturbances in local circuits can have more disastrous effects in downstream pathways. If PDE4 is acting to modulate cAMP signalling downstream from DRD1 and NMDA receptors, then it may play a critical role in fine tuning of neuronal responses to stimuli, or even act to

compensate for small scale dysregulation in these pathways. From the results observed earlier in this thesis, DISC1 may also modulate PDE4, perhaps as part of a dynamic signalling complex. This evidence raises the possibility of a connection between two well established theories of schizophrenia pathophysiology and two genetic risk factors, DISC1 and PDE4. If DISC1 and PDE4 are acting downstream of receptors to fine tune cAMP fluctuations, then it is easy to understand how changes in their function, such as those arising from non synonymous SNPs and different protein binding profiles, may lead to disarray in cAMP signalling. In the paragraph below I describe evidence suggesting that fine control of cAMP downstream of GPCRs is critical for dendritic spine function, which serves as a useful paradigm for this hypothesis.

The Arnsten group have previously demonstrated that elevated cAMP in the prefrontal cortex (PFC) of aged rats and monkeys leads to disruption of working memory, a process which relies on transient activation of local micro circuits (Ramos et al., 2003). Similarly, spatial delay related firing (relevant to working memory) was enhanced in rat PFC by cAMP inhibition and inhibited by treatment with etazolate, a PDE4 specific inhibitor. This delay related firing was closely related to the function of Hyperpolarisation-activated Cyclic Nucleotide-gated (HCN) ion channels, with pharmacological channel blocking resulting in increased firing in control and etazolate treated rats. A working model was proposed where HCN channels open in response to elevations in cAMP, lowering membrane resistance of dendritic spines and enabling gating of glutamatergic transmission. In this model, local cAMP changes arising from activity of GPCRs such as the post synaptic α2A adrenoceptor and DRD1 can influence the excitability of axospinous inputs to cortical pyramids, with an excess of cAMP leading to disconnection of these circuits. Indeed, pharmacological inhibition of HCN channel activity enhanced local network activity in ferret PFC slices and improved the performance of rats in the spatial delayed alternation task, a measure of spatial working memory. HCN channels were localised to the base of emerging spines, and perisynaptically around excitatory synapses in spine heads in post mortem monkey PFC tissue (Wang et al., 2007). It is no great leap to suggest that the PDE4s and DISC1 may

also be acting in these locations, to fine tune the control of cAMP fluctuations. Indeed, DISC1 has been localised to dendritic spines in post mortem human tissue (Kirkpatrick et al., 2006), and colocalises with HCN channels in monkey PFC (Paspalas and Arnsten, personal communication).

This model is one demonstration of how dysfunction of DISC1 or PDE4 may alter the physiological function of cortical circuitry, and predispose to aspects of psychiatric disease. In my experiments I have shown the potential for PDE4 to be acting downstream of the NMDA and DRD1 receptor, both being of major interest to psychiatric disease. A major criticism of both the dopamine and glutamine theories of schizophrenia is that schizophrenia is a relatively mild disease compared to the more widespread disarray you would expect from a large scale disruption in neurotransmitter signalling. The presence of these two schizophrenia risk associated proteins fine tuning the response downstream of NMDA and DRD1 is an attractive addition to these theories, offering a potential means by which a more subtle and localised disease may result from a larger scale signalling disruption.

The dendritic spines, structures which NRI, DRD1, PDE4 and DISC1 have been localised to (Bradshaw et al., 2008), have a small cytoplasmic volume, making them exquisitely sensitive to cAMP fluctuations downstream of these receptors. Poor control of cAMP in these structures may lead to loss of normal neuron function (Wang et al., 2007) through aberrant excitation and CRE related gene transcription. Very recent evidence suggests one role for DISC1 in dendritic spines may be modulation of their size, AMPA receptor expression, and miniature Excitatory Postsynaptic Currents (mEPSC) activity downstream of NMDA receptor activity via interactions with Kalirin-7 and Rac-1 (Hayashi-Takagi et al.). Rac-1 activation has been shown to be cAMP responsive in growth cones of rat neuroblastoma cells, with application of the degradation resistant 8-bromo-cAMP resulting in Rac-1 activation (Seifert et al., 2009), Thus, local cAMP concentrations and PDE4 activity changes may also affect the functioning of this pathway to alter dendritic spine function.

Finally, evidence from the T1:11 (DISC1) and T1:16 (PDE4B) lymphoblastoid cell lines suggests that both translocations result in decreased expression of the interrupted protein (Millar et al., 2005b). In patients carrying these translocations, a sub optimal level of DISC1 and PDE4B may lead to aberrant regulation of cAMP signalling in dendritic spines, potentially resulting in related changes in gene expression and functional connectivity of neural circuits. These changes may predispose to psychiatric disease such as schizophrenia and psychosis.

7 Chapter 7: Concluding Remarks

7.1 Studying DISC1 and intracellular signalling

Over the last ten years, DISC1 has become accepted as one of the most intensively studying and insightful schizophrenia susceptibility genes. Evidence has accumulated on many levels, genetic, behavioural, and *in vitro*, linking DISC1 to psychiatric disease. Knowledge about DISC1 has also led to increased interest in the proteins it interacts with, highlighting new or "out of fashion" pathways for refocused research. Of particular interest to this project is the dynamic and isoform specific interaction between DISC1 and the PDE4 enzymes, and the interaction between DISC1 and GSK3β. These proteins are intricately involved in intracellular signalling pathways, through control of cAMP fluxes and phosphorylation respectively. For this reason a hypothesis was formed that DISC1 may be involved in modulating intracellular signalling pathways, through its interaction with these two proteins.

In the opening chapter, a plan was set out for investigating this hypothesis. This plan had three aims, which were: to define a suitable cellular system for assessing PDE4 activity, to assess the effect of DISC1 overexpression and knock down on PDE4 activity in this system, and finally, to assess the use of PDE activity assays downstream of receptor signalling in neurons. To recap, each of these points will be briefly addressed in these concluding remarks, before speculating on the wider implications of this modest work. To end this section, I will suggest future work that will begin to answer some of the questions posed by these experiments.

7.2 Defining a suitable cellular system for assessing PDE4 activity

SHSY5Y cells were an obvious first choice of cell line for this study, being of neuronal origin (neuroblastoma) and widely used by the research community. SHSY5Y cells were found to possess a suitable, relatively high level of endogenous PDE4 specific

activity approximately three times that measurable in HEK293 cells. Overexpression and knockdown of DISC1 had no effect on expression of PDE4 mRNA or protein as measured by quantitative RT PCR and western blot. Overexpression of DISC1 however, did lead to an accumulation of PDE4B and PDE4D protein in the nucleus.

As expected from the literature, treatment of SHSY5Y cells with forskolin led to significant cAMP accumulation and a significant increase in PDE4 activity, as measured by the AlphaScreen and PDE activity assays respectively. Perhaps unexpectedly, treatment with lithium chloride and the GSK3β specific inhibitor SB216763 also affected PDE4, leading to a consistent and replicable reduction in activity. This suggests a previously undescribed tonic activating effect of GSK3β on PDE4 activity.

7.3 The effect of DISC1 on PDE4 activity

In all but one of the experiments performed, DISC1 overexpression or knockdown had no effect on basal levels of PDE4 activity. At high passage numbers, PDE4 activity decreased in DISC1 knockdown cells in comparison with control cells of an equal passage number, suggesting a long term adaptive response to DISC1 loss was occurring. Both DISC1 overexpression and lithium treatment prevented the forskolin stimulated increase in PDE4. This suggests that lithium either blocks PDE4 activity directly, or that GSK3β is also necessary for the PDE4 activity increase to occur. This response to forskolin has been previously described as PKA dependant, but the requirement for either enzyme is by no means mutually exclusive. DISC1 may be acting in the same mechanism as lithium, through inhibition of GSK3β, or inhibiting the phosphorylation of PDE4 by PKA. Counter-intuitively, knock down of DISC1 also partially inhibits the forskolin stimulated increase in PDE4 activity, suggesting the requirement for DISC1 to be present as part of a complex, potentially bringing other proteins together than may be required for increasing PDE4 activity.

Overexpression of DISC1 also affected the response of PDE4 to lithium treatment. The presence of excess DISC1 augmented the decrease in PDE4 activity. This may be due to context dependent inhibition of GSK3β by DISC1, or an indirect effect on GSK3β, via AKT activation for example. Fittingly, DISC1 knockdown did not alter the magnitude of the PDE4 response to lithium. Finally, localisation of overexpressed tagged GSK3β and PDE4B does not change with regard to DISC1 expression status.

7.4 PDE4 signalling downstream of NMDA and DRD1 receptors

The presence of all cAMP PDEs except PDE4C was established by PCR in mouse primary cortical neurons. The signal obtained from the PDE3B and PDE8A primer pairs was weak compared to the signal obtained from a positive whole brain control. Using isoform specific inhibitors, basal activity of all cAMP PDEs was also established. Primary cortical neurons were treated with a combination of NMDA and glutamate to agonise NMDA receptors, and the DRD1 specific agonist A68930. The PDE4 responses to these agonists were variable in magnitude, but despite this challenge, a significant increase in PDE4 activity in response to 2 and 5 minutes treatment with NMDA was established, and a trend (limited by the number of experiments) towards an increase was observed upon DRD1 agonism. Furthermore, colocalisation of PDE4B and D was observed with the NR1 subunit of the NMDA receptor, and DRD1 in developing dendritic spines. These results suggest a critical role for PDE4, and by extension of earlier results presented in this thesis, DISC1, in inactivating cAMP fluctuations downstream of these receptors, both of which are strongly linked to pharmacological theories of schizophrenia.

Taken together, these results suggest that DISC1, PDE4 and GSK3β may act to fine tune cAMP signalling downstream of extracellular signals, such as the NMDA receptor and GPCRs. While DISC1 is not essential for PDE4 activity changes to occur, my results demonstrate that it does modulate the PDE4 response to both forskolin and lithium. This opens up new and productive avenues for research that link long standing observations

that lithium is a potent mood stabiliser, with a potential mechanistic explanation that involves independently established genetic risk factors. There is convincing evidence, including the dopaminergic and glutamatergic theories of schizophrenia, that the majority of the symptoms of schizophrenia can be explained by aberrant activity in connected neural circuits, probably as a result of synaptic dysfunction. Synapses are immensely complex multi-protein arrangements. The post synaptic proteome may comprise over 1000 proteins, including several extracellular receptors (Collins et al., 2006). A large number of these extracellular receptors are coupled to adenylyl cyclase, and thus signal via cAMP. The presence of PDE4s allows for containment of cAMP fluxes downstream of these receptors, and their control by PKA and ERK allows integration of activity from a series of active intracellular pathways. Fine control of cAMP fluxes is particularly important in dendritic spines, where small changes can cause large effects due to their low cytoplasmic volume. My experiments suggest that DISC1 and GSK3β can also act to modulate PDE4 signalling, allowing an extra level of control to "fine tune" PDE4 output and thus cAMP fluxes. To speculate, small changes in DISC1 and PDE4 sequences, such as the presence of non-synonymous risk SNPs, may result in differential expression of protein isoforms, differential localisation of protein, or an altered protein binding profile. This may result in loss of cAMP fine tuning, leading to aberrant local gene expression or neuron excitability, resulting in a domino effect of inappropriate activity in connected circuits, and predisposing to psychiatric disease.

To continue this theme, it is likely that DISC1, GSK3β and the PDE4s are acting as part of a multi-component and dynamic signalling complex of which PDE4 activity changes and cAMP control form one of multiple outputs. Differentially localised isoforms of hub proteins such as DISC1 may vary their profile of binding proteins depending on cellular localisation, and in response to changing cellular cues. This dynamic complex-formation would allow for exquisitely tight control of intracellular signalling in response to extracellular cues, and the presence of proteins carrying risk variants may therefore lead to an altered protein binding profile, disrupting tightly controlled pathways and

predisposing to psychiatric conditions. This hypothesis suggests an agreement with the genetic theory of multiple common genetic variants with small effects predisposing to psychiatric disease. Possession of single risk variants may be compensated for by activity changes in associated pathways. Possession of multiple risk variants in associated pathways however, most likely along with an environmental contribution, may result in dysregulation that rises above a disease threshold, in unable to be compensated for, and results in psychiatric disease. While all the pathways covered in this thesis are closely integrated, possession of specific risk variants may dictate dysregulation in specific brain locations, or a subset of these central pathways, thus dictating the manifestation of the disease. A small scale proof of this concept is the different behavioural phenotypes observed in the DISC1 ENU mutants. This may eventually lead to the ability to genetically diagnose bipolar disorder or schizophrenia, or perhaps more likely, result in the reclassification of psychiatric disease into genetic subtypes, as is beginning to be done successfully with other complex diseases, such as breast cancer (Huber et al., 2009, Sørlie et al., 2003). The final goal of such reclassification would be the development of personalised medicine, designed to target the pathways affected by specific risk variants.

7.5 Future work

The experimental evidence described in this thesis is tantalising, but these early proof-of-concept experiments require further mechanistic elucidation and extra experimentation to assess the true biological significance of these events. Many of the further experiments I will suggest require genetic manipulation of cell lines. As discussed in Chapter 3, the process of transient transfection altered PDE4 activity, and so the use of stable cell lines should be considered. Issues which should be addressed with some priority are:

1. More evidence is required to confirm that lithium is acting on PDE4 through GSK3β. I have shown evidence that pharmacological GSK3β inhibition

decreases PDE4 activity to the same magnitude as lithium, but this does indicate a direct effect of GSK3β. The hypothesis would be substantiated if the effect of lithium on PDE4 activity was ablated by knocking down GSK3β. However, as there are several targets of GSK3β, knockdown may result in changes in other pathways, such as PKA signalling and Wnt signalling, that may further impact on PDE4 activity. Such changes would need to be carefully controlled for.

2. It would be interesting to assess PDE4 activity in GSK3β knockout mice, to determine whether compensatory long term changes in PDE4 activity occur in response to a lack of activation from GSK3β.

3. The relationship between PKA and lithium treatment also needs further investigation. Again, GSK3β knock down cells could be used to assess whether GSK3β is necessary for the PKA mediated increase in PDE4 activity to occur, or whether lithium simply blocks PDE4 activity from the outset.

4. It is presently unclear whether lithium treatment affects all PDE4 isoforms. Isoform selective knock-downs in cells could be used to assess the effect of lithium on PDE4A, PDE4B or PDE4D. However, it is perhaps more likely that subsets of these major isoforms are sensitive to lithium treatment. For example, PKA phosphorylation only occurs on long isoforms, and ERK phosphorylation has different effects on long and short isoforms (MacKenzie et al., 2002, Baillie et al., 2000). In Mackenzie *et al*, 2002, the effect of PKA on different PDE4 isoforms was examined by overexpression of various subtypes. A cell-line with low endogenous PDE4 activity, such as HEK293, would be ideal for such an experiment involving lithium.

5. Various experiments have shown conflicting evidence for an effect of DISC1 expression status on AKT and ERK phosphorylation. It would therefore be useful to know if the drugs treatments and expression changes in these

experiments altered their phosphorylation status. This could be examined by use of western blotting with phospho-antibodies. This was briefly attempted as part of the PhD thesis, but the phospho-antibodies did not work well with lysates made from 3T3 buffer, and thus the experiments would have to be re-run.

6. The potential for GSK3β to directly phosphorylate PDE4 should also be investigated. As detailed in the conclusion of Chapter 4, this may be a difficult experiment to perform due to the general requirement for priming phosphorylation of GSK3β targets to achieve high efficiency phosphoryation. It may be possible to drive phosphorylation by using high concentrations of recombinant GSK3β in *in vitro* or high-throughput array approaches. At present there is no clear candidate for a putative GSK3β phosphorylation site. If only certain PDE4 isoforms are found to be responsive to lithium treatment (see point 3), then this may enable candidate sites to be refined, and a traditional site directed mutagenesis approach to phosphorylation detection to be performed (Scott, 2006).

7. As with lithium treatment, it may be useful to further define which PDE4 isoforms are activated downstream of NMDA and DRD1 signalling. This may be achieved by low throughput PDE assays in neurons, or indirectly through cAMP measurement in brain slices (Suvarna and O'Donnell, 2002). Allosteric modulators which show preferential inhibition of PDE4D over PDE4A, 4B and 4C are in development (Burgin et al., 2010), and may be useful in this paradigm. Alternatively, neurons from mice with isoform specific knockdown may be performed. If PDE4D is the major PDE4 responsible for cAMP inactivation downstream of NMDA, for example, then brain slices from PDE4D knock out mice would show an accumulation of cAMP after NMDA treatment, similar to the effect seen on rolipram or IBMX application.

8. It was interesting to see an accumulation of PDE4B and PDE4D in the nucleus when DISC1 was overexpressed. A similar effect has been observed in an experiment with GSK3β, which despite possessing a nuclear localisation signal is mostly found in the cytoplasm. The authors of this study proposed that GSK3β is anchored in the cytoplasm by other proteins, which are saturated when GSK3β is overexpressed, resulting in a nuclear localisation of overexpressed protein (Meares and Jope, 2007). It is possible that this may be occurring with DISC1 in my experiment. For this reason, it would be interesting to assess the localisation of endogenous GSK3β with respect to the PDE4s and DISC1. My experiment involving exogenous tagged protein may saturate a potential effect on protein localisation.

9. It is highly unlikely that DISC1, PDE4 and GSK3β are the sole components of the functional complex. Instead, they are likely to function as part of a dynamic, multi-component complex. It would be wonderful, (if expensive, at present!) to be able to assess the composition of such a complex, and the changes that might occur in it on a large scale, in response to forskolin stimulation for example. Stable isotype labelling with animo acids in cell culture (SILAC) can be used in combination with chemical cross linking, to allow for quantitative assessment of non-covalent links between proteins under different cellular conditions by mass spectrometry (Trakselis et al., 2005, Bose et al., 2006, Guerrero et al., 2006). In their 2006 paper, Bose *et al* use this system to assess proteins phosphorylated by tyrosine kinase as a result of Her2 overexpression. In this experiment, three cell cultures were maintained on media containing three different weight arginine isotypes. These cultures were subjected to different conditions, in this example Her2 overexpression and tyrosine kinase inhibition. Cultures were then lysed, the lysates combined, and immunoaffinity purified using an antiphosphotyrosine antibody. Purified proteins were trypsinised, then quantitatively assessed by liquid chromatography mass spectrometry (LC-MS). Peptide peaks obtained by LC-MS are shifted by the different weight of the arginine residues. To identify

the proteins involved, peptide sequences were compared with protein sequence databases. A scheme for this experiment is shown in Figure 2.A of Bose *et al*, 2006.

Thus, one can envisage an experiment where SHSY5Y cells may be treated with forskolin or vehicle, then chemical cross linked, and a lysate produced. DISC1 interacting proteins may then be immunoaffinity purified using a DISC1 antibody, trypsinised and quantitatively analysed by LS-MS. These type of experiments could also be used to assess the effect of overexpression of full length or mutant proteins, or knock down of proteins, on the composition of complexes.

On a smaller scale, the interaction between PDE4s and DISC1 has already been assessed by immunoprecipitation with respect to forskolin stimulation. It would be interesting to add GSK3β overexpression and lithium treatment to such an experiment. Unpublished work from our laboratory finds no evidence for competitive binding between GSK3β and PDE4B with DISC1, and moreover that the GSK3β-DISC1 interaction is weak (Ogawa *et al*, unpublished).

10. Finally, as detailed in the conclusion of chapter 6, it would be beneficial to develop a cellular system that would measure PDE4 activity with increased spatial resolution. As previously discussed, these include genetically encoded FRET based cAMP sensors, based on PKA and EPAC proteins and cyclic nucleotide gated ion channels (Houslay, 2010). It may even be possible to employ optogenetic techniques to assess cAMP fluctuations and gene expression in DISC1 or PDE4 isoform mutant mice, in response to excitation of neurons by light responsive microbial opsins (Gradinaru et al., 2007). The low throughput assay system was unable to detect a decrease in PDE4 activity in neurons in response to lithium treatment, but it remains unclear as to whether this is because it does not occur, because the assay is not sensitive enough to detect a decrease

in a low basal rate of activity, or because PDE4 activity changes are a localised response compensated for elsewhere in the cell.

This list of potential future directions is clearly not comprehensive, and it is clear that this preliminary work asks many more questions than it answers. These are the approaches however, that I believe would be the most informative in the short term, in order to direct future research down productive avenues.

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Supplementary Material 1 - Materials

S.1 Solutions and buffers

Below are listed the details for making up all of the solutions for the methods described in Chapter 2 – Materials & Methods.

S.1.1 Poly D-Lysine solution

Used for coating coverslips and plates prior to primary neuron seeding.

25ml	0.3M Borate buffer (Sigma)
22.5ml	dH_2O
2.5ml	500µg/ml Poly D-Lysine (Sigma)

S.1.2 Dissection Buffer

Used to dissect foetal mouse brains in primary neuron production. All reagents from Gibco.

500ml	HBSS with $CaCl_2$ & $MgCl_2$
5ml	L-Glutamate
3.5ml	HEPES (cell culture standard, pH7.3-7.5)

When trypsinising dissected brain tissue, 20mls of TrypLE is added to 30mls dissection buffer.

S.1.3 Neurobasal Medium

Growth medium for primary neuronal cultures. All reagents from Gibco.

500ml	Neurobasal medium
10ml	B27 supplement
5ml	GlutaMAX-1 supplement
2.5ml	Penicillin/Streptomycin solution

S.1.4 RIPA Lysis Buffer

Used to lyse cultured cells in preparation for Western blotting.

2.5ml	1M Tris-HCL pH 7.5 (Fisher)
2.5ml	3M NaCl (Fisher)
0.5ml	IGEPAL (Sigma)
2.5ml	10% Sodium deoxycholate (Fisher)
250µl	20% SDS (Fisher)

Make up to 50mls with dH_2O. Store at -20°C. Before being used in lysis protease inhibitor cocktail (1 tablet dissolved in 2mls dH_2O, Roche) must be added at a concentration of 1 in 25.

S.1.5 3T3 Lysis buffer

To lyse cultured cells and primary neurons for use in phosphodiesterase assays.

1.25ml	1M HEPES (Gibco)
0.5ml	5M NaCl (Fisher)
5ml	0.5M NaFl (Fisher)
5ml	Glycerol (Promega)
0.5ml	Triton X-100 (Sigma)
3ml	0.5M Sodium pyrophosphate (Fisher)
50µl	1M DTT (Sigma)
625µl	0.2M EDTA (Sigma)

Make up to 50mls with dH_2O. Store at -20°C. Before being used in lysis protease inhibitor cocktail (1 tablet dissolved in 2mls dH_2O, Roche) must be added at a concentration of 1 in 25, and Protease Inhibitor II (Calbiochem) at a concentration of 1 in 200.

S.1.6 Protein Sample Buffer

To denature proteins and prepare lysates for Western Blotting.

6.25ml	1M Tris pH 6.8 (Fisher)
10ml	Glycerol (Promega)
10ml	20% SDS (Fisher)
13.75ml	dH_2O
0.5mg	Bromophenol blue (Sigma)

Store at room temperature.

S.1.7 Western blot Running buffer

To run Western blots on Invitrogen pre poured gels.

20ml	20x NuPage Tris Acetate SDS Running Buffer (Invitrogen)

Make up to 400mls with dH_2O just prior to use.

S.1.8 Western blot transfer buffer

To transfer Western blots from Invitrogen pre poured gels onto Invitrolon PVDF membrane.

25ml	20x NuPage Transfer Buffer (Invitrogen)
50ml/gel	Methanol (Fisher)

Make up to 500mls with dH_2O just prior to use.

S.1.9 Western Blot Wash buffer

Used for washing membranes during Western blotting, and for dilution of the secondary antibody.

100ml	10x PBS (Sigma)
900ml	dH_2O
2ml	TWEEN-20 (Sigma)

S.1.10 Ponceau S stain

To visualise successful transfer of protein to the membrane during Western Blotting

1g	Ponceau S (Sigma)
4ml	Acetic Acid (Fisher)

Make up to 200ml with dH_2O. Solution is stored at room temperature, and can be reused after staining if returned to an appropriate container.

S.1.11 Membrane Blocking Buffer

Membrane blocking buffer is use to block western blots pre staining, and also to dilute the primary antibody for application.

2.5g	Instant Milk Powder (Marvel)
5ml	10x PBS (Sigma)
100µl	TWEEN-20 (Sigma)

Make up to 50ml with dH_2O, and store overnight at 4^oC if required. Solution should not be kept for longer than this.

S.1.12 Mowiol mounting solution

Used to mount slides for immunocytochemistry, contains DAPI (Sigma) to visualise nuclei and DABCO to decrease fading of staining.

7.5g	Mowiol (Sigma)
10ml	Glycerol (Promega)
25ml	dH_2O

Solution left overnight at room temperature.

50ml	0.2M Tris-HCL pH 8.5 (Fisher)

The resulting solution is boiled to 100^oC for 20 minutes and left to cool.

1.75g	DABCO (Sigma)

The final solution can be stored at $-20\ ^oC$. Working aliquots with DAPI added to a final concentration of 50µg/ml can be stored at $4\ ^oC$ for up to six months.

S.1.13 dNTP mix

An equal mix of PCR grade ATP, CTP, GTP and TTP (PCR grade, Sigma) were diluted to 5mM in 1mM Tris pH 7.5 (Fisher) for use in PCR.

S.1.14 TBE Buffer (5x)

Dilute to 0.5x for preparing and running agarose gels.

54g	Tris Base (Sigma)
27.5g	Boric Acid (Sigma)
20ml	0.5M EDTA (Sigma)

Make up to 1 litre with dH_2O. Store at 4^oC.

S.1.15 Stop solution

Used to load PCR products onto agarose gels for electrophoresis.

3g	Ficoll 400 (Sigma)
50µl	20% SDS (Fisher)
800µl	0.5M EDTA (Sigma)
25µg	Orange G (Sigma)

Make up to 1 litre with dH_2O. Solution was stored at room temperature.

S.1.16 2xYT medium

Used to amplify bacterial cultures immediately post transformation.

16g	Tryptone (Sigma)
10g	Yeast Extract (Sigma)
5g	Sodium Chloride (Fisher)

Adjust the pH to 7.0and make up to 1 litre with dH_2O. Store at 4^oC.

S.1.17 L Agar/ampicillin

L agar is used to prepare plates for selection of transformed E-coli bacteria.

50 g	Tryptone
25 g	Yeast Extract
50 g	Sodium Chloride

Make up to 5 litres with dH_2O, and adjust pH to 7.2. Pour into a bottle and add 75g of Agar. Store at 4°C until use. Melt at a moderate temperature in a microwave until a homogenous clear brown solution is obtained. Leave to cool until the flask is touchable with gloved hands. Add ampicillin to a final concentration of 50µg/ml, and pour into plates immediately.

S.1.18 L Broth/ampicillin

L broth is used to amplify bacterial cultures after transformation and growth on L Agar plates.

50g	Tryptone
25g	Yeast Extract
25g	Sodium chloride

Make up to 5 litres with dH_2O, and adjust pH to 7.2. Store solution at 4°C. Immediately prior to use add ampicillin to a final concentration of 50µg/ml.

S.1.19 PDE Assay Tris/Mg^{2+} buffer

To dilute tritiated cAMP for PDE assays.

400µl	1M TrisHCl pH7.4 (Fisher)
200µl	1M $MgCl_2$ (Sigma)

Make up to 20mls with dH_2O and add:

40µl	1mM cAMP (Sigma)
60µl	[^{3}H] cAMP (Perkin Elmer)

Vortex well, and aliquot in 1ml amounts and store at -20°C.

S.1.20 Dowex Slurry

The ion exchange resin used in PDE Assays

400g	Dowex Mesh CL (Sigma)
4l	1M NaOH (Fisher)

Stir for 15 minutes, then wash 30 times with dH_2O until pH7 is reached. Add:

4l	1M HCl (Fisher)

Stir for 15 minutes, then wash 5 times with dH_2O until pH3 is reached. Store as 1:1 slurry in dH_2O at 4^oC.

S.1.21 AlphaScreen control buffer

Used to generate a cAMP standard curve in AlphaScreen quality control.

1ml	10x control buffer (Kit component: 5mM HEPES, 50mM NaCl)
100µl	3% Tween-20 (Kit component)
8.9ml	dH_2O

Make up immediately before use.

S.1.22 AlphaScreen Stimulation Buffer

The agonist/tested drug is diluted in this buffer in the first stage of the AlphaScreen assay.

35ml	1x indicator free HBSS (Gibco)
4ml	1% BSA (Sigma)
40µl	500mM IBMX (Sigma)
200µl	1M HEPES (Gibco)

Adjust to pH 7.4 with 0.1N NaOH (Fisher). Prepare immediately before use.

S.1.23 AlphaScreen Lysis Buffer “Detection mix”

To contain the donor beads in the second stage of the AlphaScreen assay.

17ml	dH_2O
2ml	1% BSA (Sigma)
600µl	10% Tween-20 (Sigma)
100µl	1M HEPES (Gibco)

Adjust to pH 7.4 with 1N NaOH (Fisher). Prepare immediately before use.